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COMPARISON OF FRESH, LIQUID-PRESERVED, AND CRYOPRESERVED  
PLATELETS:  
CHANGES IN THROMBOXANE A2 PRODUCTION, ADHESIVE SURFACE RECEPTORS,  
AND MEMBRANE PROCOAGULANT ACTIVITY

BY

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## ABSTRACT

Previous studies have determined that compared to 3-4 day 22°C liquid-preserved platelets, transfusion of cryopreserved platelets results in improved hemostatic function despite decreased survival in the setting of cardiopulmonary bypass. We studied fresh, 2-4 day stored liquid-preserved platelets, and cryopreserved platelets using monoclonal antibodies directed against platelet surface CD62P, glycoprotein (GP) Ib, activated GPIIb-IIIa complex, and surface-bound coagulation factor V by a 3 color flow cytometric method. Platelet production of thromboxane B<sub>2</sub> (TXB<sub>2</sub>) was measured by RIA after aggregation with a combination of arachidonic acid and ADP. Whereas the production of TXB<sub>2</sub> by liquid-preserved platelets after stimulation with ADP/arachidonic acid combination was significantly lower ( $161 \pm 224$  pg  $\times 10^{-6}$ /platelet) than fresh platelets ( $346 \pm 202$ ,  $p = 0.02$ ), the production of TXB<sub>2</sub> by cryopreserved platelets after stimulation was significantly higher ( $589 \pm 341$ ) than fresh platelets ( $353 \pm 215$ ,  $p = 0.02$ ). Compared to fresh or liquid-preserved platelets which had >97% normal surface levels of GPIb, cryopreserved platelets were composed of distinct subpopulations of 51.4% GPIb-normal and  $48.6 \pm 9.1\%$  (mean  $\pm$  SD) GPIb-reduced platelets. Both populations exhibited a significantly increased percentage of procoagulant platelet-derived microparticles compared to fresh or liquid-preserved platelets, based on decreased forward light scattering and bound factor V ( $12.3 \pm 7.5\%$  of the GPIb-normal and  $95.3 \pm 1.9\%$  of the GPIb-reduced,  $p < 0.02$  compared to fresh,  $0.4 \pm 0.2\%$ ). Total binding of factor V was also elevated in the post-thaw-washed cryopreserved platelet product compared to either fresh or day 3/4 liquid-preserved platelets with the GPIb-reduced population accounting for the majority of this activity. Platelet surface activated GPIIb-IIIa was elevated on the GPIb-normal subpopulation of cryopreserved platelets, but not on the GPIb-reduced subpopulation. Only the GPIb-normal population retained activated GPIIb-IIIa responsiveness to in vitro agonist addition. Surface P-selectin, indicating degranulation, was elevated in both subpopulations of cryopreserved platelets, but only the GPIb-normal population retained P-selectin responsiveness to in vitro agonist addition. Day 3/4 liquid-preserved platelets had a more modest increase in surface P-selectin, and no increase in activated GPIIb-IIIa. These platelets retained agonist responsiveness similar to that of the fresh platelets. The observed hemostatic effectiveness of cryopreserved platelets compared to liquid-preserved platelets after cardiopulmonary bypass may be due to delivery of procoagulant activity and thromboxane to bleeding sites.

## INTRODUCTION

A recent prospectively randomized double-blind study showed reductions in transfusion requirements and non-surgical blood loss following complex cardiopulmonary bypass surgery when cryopreserved platelets were used compared to 3-4 day 22°C liquid preserved platelets.<sup>1</sup> This improved hemostasis occurred despite a decreased platelet survival in the group, suggesting the cryopreserved platelets were functioning immediately to bring about cessation of bleeding.

Whereas the quality of a platelet transfusion is often judged by the product's ability to increase the platelet count, there are many circumstances when consumption of an altered platelet product to shorten an acute bleeding episode would be beneficial.

Storage induced morphological changes and decreased responsiveness to in vitro agonists is presumed to be deleterious because they compare unfavorably with fresh platelet preparations in tests such as aggregation and recovery from hypotonic stress.<sup>2</sup>

However, platelets which have undergone activation processes and have lost or altered agonist receptors (and therefore reactivity) may possess characteristics which predispose them to immediate hemostatic functioning.

Cryopreserved platelets have a number of these "defects" which has lead many investigators to dismiss them as having an unacceptable "platelet storage lesion". Nevertheless, the important observation of reduced non-surgical bleeding was made with the use of previously-frozen platelets in a patient group with a typically high rate of transfusion requirements.<sup>1</sup> The result was to require less red cell and fresh frozen

plasma transfusions. This surprising result benefits the patient by reducing their potential exposure to bacterial and viral infection.

The question then becomes: why would cryopreserved platelets which are demonstrably less reactive in vitro with a decreased survival in vivo deliver improved hemostasis compared to the liquid preserved platelets currently in widespread use? This study was undertaken to identify properties which are likely candidates to explain this result.

## METHODS

Plateletpheresis Procedure: Plateletpheresis products were collected at the Naval Blood Research Laboratory from normal donors meeting the requirements of AABB for healthy blood donors. None of the participants had taken medication for ten days prior to donation. Samples of peripheral blood were drawn from the donor in sodium citrate (Vacutainers, Becton-Dickinson, San Jose CA) prior to plateletpheresis. Platelets were collected using the Haemonetics Mobile Collection System (MCS, Haemonetics, Braintree, MA) and ACD formula A anticoagulant. The products were sampled and put into 1000 ml CLX (tri-[2-ethyl-hexyl] trimellitate polyvinylchloride) platelet storage bags (Baxter, Deerfield, IL). For liquid preservation, the plateletpheresis product was placed on an Eberbach shaker at 140 oscillations per minute at 22°C for up to 6 days. Platelet products which were cryopreserved were stored at 22°C with agitation for the initial 24 hour period.

Platelet Cryopreservation: The cryopreservation method used for this study has been previously described in detail.<sup>3</sup> Briefly, the 24 hour plateletpheresis products were transferred into a 1000 mL PVC platelet freezing bag (Fenwal 4R2986, Deerfield, IL). A volume of 50 mL of 27% DMSO in saline was added at room temperature over a 5 minute period to achieve a final concentration of 6% DMSO. The platelets were placed in an aluminum container and kept at -80°C to achieve a freezing rate of 2-3°C per





Platelet Aggregation in Response to 0.5 mg/mL Arachidonic Acid and 0.2  $\mu$ M ADP:

Platelet aggregation was measured at 37°C using the Biodata aggregometer and Biodata reagents (Biodata Corp., Hatboro, PA). The plateletpheresis product was adjusted to a platelet count of approximately  $5.0 \times 10^8$ /ml. A 50  $\mu$ l combination of 5 mg/mL arachadonic acid and 2  $\mu$ M adenosine diphosphate (ADP) was added to 450  $\mu$ l of diluted platelets (0.5 mg/mL arachadonic acid and 0.2  $\mu$ M ADP final concentrations).

The platelet aggregation pattern was recorded for 5 minutes. The 5 minute aggregation patterns were analyzed by digitizing the area under the curve using the Kurta Is/ONE input system and the data reported as digitizer units for the five minute period of aggregation.

Thromboxane B<sub>2</sub> Assay: Thromboxane B<sub>2</sub> (a stable breakdown product of thromboxane A<sub>2</sub>) was measured using a commercially available radioimmunoassay (New England Nuclear, Boston, MA) and used according to the manufacturers instructions. The presence of thromboxane B<sub>2</sub> in the plasma of plateletpheresis products is an indicator of platelet thromboxane synthase activity which occurs with platelet activation . In this study the level of thromboxane in the fresh and 24 hour stored plateletpheresis products were measured in the cell free plasma, prepared by centrifuging the sample at 1650 x G and 4°C for 10 minutes. The cell free plasma samples were frozen at -80°C until assayed.

In addition, platelet production of thromboxane B<sub>2</sub> per platelet was measured in the supernatant after aggregation with a combination of 0.5 mg/mL arachadonic acid

and 0.2  $\mu$ M ADP. After 5 minutes of aggregation, ibuprofen (0.02 ng/mL final concentration) was added to the cuvette to arrest thromboxane production and placed on ice. The sample was then centrifuged (1650 x G, 4°C) and the plasma frozen at -80°C until assayed as described above. An unaggregated sample was treated identically to establish baseline thromboxane B<sub>2</sub> data. Platelet counts were performed on samples of the initial PRP used for aggregation and adjusted for dilution by the agonist and ibuprofen additions. The thromboxane production per platelet was calculated as follows:

$$\text{Thromboxane production per platelet (in picograms)} = \frac{\text{Aggregated TXB}_2 \text{ Value (pg)} - \text{Baseline TXB}_2 \text{ value (pg)}}{\text{Platelet Count / 0.1 mL}}$$

Plasma pH Assay: The plasma pH was measured at 37°C on the IL Blood Gas Manager 1312 (Instrumentation Laboratories, Lexington, MA) in non-diluted samples drawn into a tuberculin syringe, capped tightly. Samples were run within 5 minutes, or kept on wet ice for up to 2 hours.

Preparation for flow cytometry: These methods are essentially as previously described for whole blood flow cytometry.<sup>4,6</sup> There were no centrifugation, gel filtration, vortexing, or stirring steps that could artefactually activate platelets. The following monoclonal antibodies were used: 6D1 (anti-GPIb, a gift from Dr. Barry Coller) was FITC-

conjugated with the Boeringer-Mannheim "Quick-Tag" Kit, V237 (anti-factor V, a gift from Dr. Charles Esmon), PAC1 (anti-fibrinogen receptor, purchased from the University of Pennsylvania Cell Center), S12 (anti P-selectin, purchased from Centocor Inc.). Mouse IgG and mouse IgM isotypic controls (purchased from Sigma Chem. Co.) were biotinylated in our lab as previously described <sup>4</sup> and anti-CD41-phycoerythrin was purchased from Immunotech. Streptavidin-RED670 was purchased from GIBCO-BRL (Grand Island, NY). Gly-Pro-Arg-Pro (GPRP) was purchased from Calbiochem (La Jolla, CA). Calcium ionophore A23187 and calcium chloride were purchased from Sigma. Sodium citrate vacutainers were purchased from Becton-Dickinson (San Jose, CA). ADP and epinephrine was purchased from Biodata (Hatboro, PA). Human alpha-thrombin was a generous gift from Dr. John Fenton. Equine tendon collagen was purchased from Hormon-Chemie (Munich, Germany). The ultrapure methanol-free formaldehyde was purchased from Polysciences (Warrington, PA). The modified HEPES-Tyrodes buffer (H.T.) contains 137 mM NaCl, 2.8 mM KCl, 1 mM  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ , 12 mM  $\text{NaHCO}_3$ , 0.4 mM  $\text{Na}_2\text{HPO}_4$ , 5.5 mM Glucose, 10 mM HEPES and 0.35% bovine serum albumin, all buffer components were purchased from Sigma. DNA check and standard bright flow cytometry calibration beads were purchased from Coulter (Miami, FL).

Plateletpheresis unit samples or fresh sodium citrate platelet rich plasma was diluted (1:15 final concentration) in H.T. buffer containing GPRP (2.5 mM final concentration), 20% fresh sodium citrate plasma (as a source of factor V) and antibody 6D1-FITC and incubated for 10 minutes at 22°C. To this either H.T. buffer, 3 mM  $\text{CaCl}_2$ , 40  $\mu\text{M}$  A23187 / 3 mM  $\text{CaCl}_2$ , 20  $\mu\text{M}$  ADP / 20  $\mu\text{M}$  epinephrine / 3 mM  $\text{CaCl}_2$  or 2 units/mL human alpha-thrombin

/ 20 µg/mL collagen / 3 mM CaCl<sub>2</sub> (final concentration) was added and the samples incubated at 37°C for 10 minutes. To this V237-biotin or Pac1-biotin at saturating concentrations or equivalent concentrations of isotypic control MIgG or MIgM were added followed by a 10 minute 22°C incubation. Samples were then fixed by adding 1% formaldehyde for 20 minutes at 22°C and diluted 20 fold with H.T. buffer. Samples were then centrifuged for 5 minutes at 1100 x g, the supernatant removed and CD41-phycoerythrin / streptavidin-RED670 added and incubated for 30 minutes at 22°C. Samples were then diluted 20 fold and run on a Coulter EPICS XL flow cytometer. Appropriate color compensation was set for FL1, FL2 and FL3 using 525, 575 and 675 nm bandpass filters respectively. All data was saved in flow cytometry standard listmode files and analyzed using Coulter XL software. Platelets were identified based on their characteristic light scatter and binding of anti-CD41-phycoerythrin (FL2). Platelets expressing normal surface GPIb and those expressing low GPIb were easily separated based on FITC (FL1) fluorescence (see Fig 5 below) and analyzed individually. Both fresh platelets and day 3/4 liquid preserved platelets were >97% GPIb normal, therefore no analysis of GPIb low platelets was done due to an insufficient number of events. GPIb percent maximal is normalized to the binding of 6D1 to fresh platelets in the absence of added agonist. The RED670 signal (FL3) was used for analysis of the activation-dependent test antibodies Pac1, V237 and S12. In the case of V237 (directed against coagulation factor V), when the data is expressed as percent microparticles, these were defined by a triangular analysis region including a platelet-derived subpopulation exhibiting increased FL1 (V237 binding) and

decreased log forward light scatter (an indicator of cell size and refractive index). Otherwise, data is expressed as percent maximal (normalized to the maximal expression of fresh platelets to ADP/Epinephrine for Pac1 and A23187 for V237) or percent positive for the test antibody (platelets expressing fluorescence above the isotypic control histogram set at 1% of all platelets).

For analysis of platelet surface P-selectin, plateletpheresis unit samples or fresh platelet rich plasma was diluted (1:4 final concentration) in H.T. buffer containing GPRP (2.5 mM final concentration) and antibody 6D1-FITC and incubated for 10 minutes at 22°C. To this either H.T. buffer, 20  $\mu$ M ADP / 20  $\mu$ M epinephrine / 3 mM  $\text{CaCl}_2$  or 2 units/mL human alpha-thrombin / 3 mM  $\text{CaCl}_2$  (final concentration) was added and the samples incubated at 22°C for 15 minutes. Samples were then fixed by adding 1% formaldehyde for 20 minutes at 22°C and diluted 10 fold with H.T. buffer. To aliquots of this preparation, S12-biotin at a saturating concentration or the equivalent concentration of isotypic control MlgG and CD41- phycoerythrin was added and incubated for 15 minutes 22°C. This was followed by adding streptavidin-RED670 for 10 minutes at 22°C. Samples were then diluted 10 fold and run on a Coulter EPICS XL flow cytometer and analyzed as described above. Percent maximal data was normalized to the fresh platelet fluorescence after treatment with 2 units/mL human alpha-thrombin.

Single and dual parameter histograms shown in the figures below were made by importing graphics from either Isocontour<sup>®</sup> version 3.2 or WinMDI<sup>®</sup> version 2.4 histograms Sigmaplot<sup>®</sup> for Windows. All graphs and paired or unpaired T-test statistical analyses were

made using Sigmaplot® for Windows version 2. Statistical significance was accepted for p values less than 0.05.

## RESULTS

Figure 1 reports that previously frozen (cryopreserved) platelets produce significantly more thromboxane B<sub>2</sub> in response to a combination of 0.5 mg/mL arachadonic acid and 0.2 mM adenosine diphosphate than fresh plateletpheresis products. In addition, 48 hour 22°C liquid storage resulted in significantly less production of thromboxane B<sub>2</sub> after stimulation with the same agonists.

The aggregation response of these same samples is shown in figure 2. The cryopreserved platelets aggregated significantly less after activation by the combination of arachadonic acid and adenosine diphosphate than fresh platelets. Although the 48 hour 22°C liquid storage also resulted in a reduced aggregation response to these agonists compared to fresh platelet values, the p value was >0.05.

Figure 3 shows the increased sensitivity of cryopreserved platelets to hypotonic stress compared to fresh platelets. Less than 20% of the cryopreserved platelets remained intact after being exposed to the hypotonic environment. The 48 hour 22°C liquid stored platelets were able to recover from hypotonic stress as well as fresh platelets when exposed to the same conditions.

The effect of the platelet cryopreservation and 2 day liquid storage on plasma pH is shown in figure 4. Although the differences were slight, the pH of thawed-washed cryopreserved platelets resuspended in autologous fresh-frozen plasma was significantly lower than the fresh platelet preparations. The pH of the 48 hour 22°C liquid stored platelets was slightly, but significantly higher than fresh platelets.

The flow cytometer analyzes cell suspensions on a cell-by-cell basis, which makes possible the detection of subpopulations. This analytic capability is an advantage over radioisotopic methods which yield averaged results of a given cell population being studied. Figure 5 illustrates the appearance of a glycoprotein (GP) Ib reduced subpopulation of platelets (or platelet microparticles) after cryopreservation, where fresh or 3-4 day liquid preserved preparations consist entirely of platelets with a normal surface GPIb level. The separation of the GPIb-reduced and the GPIb-normal subpopulations is distinct, allowing separate analysis in the multiparameter flow cytometry assays used in this study.

Figure 6 reports quantitatively the changes in platelet surface GPIb which occur with 3-4 day liquid preservation or cryopreservation. Panel A shows that while only 2-4% of the CD41 positive platelet events has a reduced level of GPIb, nearly half of the cryopreserved platelet preparation has this reduced level of surface GPIb. Panel B reports the relative binding of the GPIb antibody, with the fresh platelet fluorescence being assigned a value of 100. The extent of the GPIb reduction can be seen in the far right bar of panel A. Less than 5% of the original platelet complement of surface GPIb accessible for antibody binding is present on the GPIb-reduced population of the cryopreserved platelet product. Comparison of these values to matched isotypic control mouse IgG samples confirmed that these were not completely negative for surface GPIb.

The histograms depicted in figure 7 illustrate the method used to quantitate percent platelet-derived microparticles. The region shown in the lower right corner



included platelets and platelet-derived microparticles exhibiting decreased log forward light scatter and increased factor V binding. As seen in panel A of figure 7, fresh platelets have very few procoagulant platelet-derived microparticles in the absence of added agonist. Panel B shows that even in the absence of in vitro added agonist, cryopreserved platelets contain a distinct subpopulation of factor V binding platelets and/or platelet-derived microparticles. Since the surface factor V expression was bimodal in distribution, both with and without agonist addition, the analysis regions established bisecting these subpopulations provided a valid method for quantitating the percent platelet-derived microparticles.

Employing the method illustrated in figure 7, we quantitated the % platelet-derived microparticles of fresh, 3-4 day liquid preserved, GPIb-normal cryopreserved, and GPIb-reduced cryopreserved platelets (Figure 8). Fresh platelets generate a modest platelet-derived microparticles population in response to 3 mM calcium ( $\text{CaCl}_2$ ) addition or 2 U/mL thrombin/20  $\mu\text{g/mL}$  collagen /3mM calcium, while having nearly no platelet-derived microparticles in the absence of agonist added in vitro. The 3-4 day liquid preserved platelets have a similar pattern of platelet-derived microparticles generation, with a slightly reduced response to thrombin/collagen/calcium addition. The GPIb-normal platelets have an increased level of factor V without agonist, and respond more vigorously to both calcium and the combined agonists. The GPIb-reduced platelets were nearly completely in the form of procoagulant platelet-derived microparticles even before addition of agonist. Since >90% of these were in the

platelet-derived microparticles form, there was little further conversion as a result of agonist addition.

Factor V binding is not confined only to the emerging platelet-derived microparticles subpopulation, but is also expressed on the platelet surface. Therefore, analysis of total procoagulant activity (factor V binding) is a useful measure for the analysis of the platelet transfusion product. Figure 9 reports total binding of anti-factor V monoclonal antibody V237 to platelets and platelet-derived microparticles which were positive for the CD41 platelet-specific identification marker. Three to four day liquid-preserved platelets were very similar to fresh platelets, with slightly more factor V binding after calcium addition than fresh. Again, the increased responsiveness of the GPIb-normal cryopreserved platelet component to calcium and thrombin/collagen/calcium can be seen as compared to fresh or liquid preserved platelets. The GPIb-reduced cryopreserved component has increased factor V binding without added agonist, which along with a reduced log forward light scatter signal, accounts for their passing the criteria for being platelet-derived microparticles as shown in figure 8. Addition of 3 mM  $\text{CaCl}_2$  caused a large increase in factor V binding to the GPIb-reduced cryopreserved population. Addition of thrombin/collagen in the presence of 3 mM calcium did not cause any further increase in factor V binding.

Figure 10 shows a set of representative flow cytometry histograms which further characterizes the factor V binding of the GPIb-normal and -reduced platelet/platelet-derived microparticles subpopulations of a cryopreserved platelet product. The GPIb-reduced population which is labeled "B" in the upper single-parameter histogram of

figure 10 has very homogenous factor V binding (see the bottom right contour histogram). In contrast, the GPIb-normal population ("A") consists of 2 distinguishable populations with high and low factor V binding (note the bottom-center histogram where there are 2 populations, each with relatively high log forward light scatter, but of differing factor V). The lower factor V binding subset in the center histogram accounts for all the lower binding events of the entire sample (see the combined histogram in the lower left). Some of the platelets in this group (normal GPIb and log forward scatter) bind very high levels of factor V.

Further application of the 3 color flow-cytometry technique examined the activated (fibrinogen binding) conformation of the GPIIb-IIIa complex and the results are reported in figure 11. Fresh and 3-4 day liquid preserved platelets functioned normally, with low levels of activated GPIIb-IIIa in the absence of added agonist (background), and maximal levels after 20  $\mu$ M ADP/20  $\mu$ M epinephrine/3 mM  $\text{CaCl}_2$ . The GPIb-normal platelets of the cryopreserved product had elevated background levels of activated GPIIb-IIIa and responded to the combined agonist, although to a lesser degree than fresh platelets. The GPIb-reduced cryopreserved platelets did not have an elevated background level of activated GPIIb-IIIa and were completely unresponsive to the added agonists. Histograms of cryopreserved platelets illustrate this point and are shown in figure 12. These are plots of GPIb on the Y axis, and activated GPIIb-IIIa on the X axis. The GPIb subsets are clearly distinguishable, and in the absence of added agonist, the higher background level of activated GPIIb-IIIa can be observed on the GPIb-normal population (upper population of panel A). After

addition of ADP/epinephrine/calcium, the GPIb-normal platelets can be seen strongly up-regulating the surface expression of activated GPIIb-IIIa (panel B). The GPIb-reduced population is unresponsive to the agonist challenge.

The role of platelet surface P-selectin (a marker of degranulation) on the 2 GPIb populations produced by cryopreservation was also examined, these results compared to fresh and 3-4 day 22°C liquid preserved platelets are shown in figures 13 and 14. Platelets positive for surface P-selectin (those with fluorescence above the irrelevant isotype-matched mouse IgG fluorescence) are reported in figure 13. Few fresh platelets have a positive P-selectin signal in the absence of added agonist, 70% become positive after 20  $\mu$ M ADP / 20  $\mu$ M epinephrine addition, and >90% are positive after 2 units/mL thrombin addition. Two to three day liquid-preserved platelet P-selectin % positive is slightly increased without agonist addition. The percent of the liquid preserved platelets responding to in vitro agonist addition is slightly reduced compared to fresh. Nearly half of the GPIb-normal cryopreserved platelet were positive for P-selectin without agonist addition, and responded to both agonist additions. The percent P-selectin positive of the GPIb-reduced population was elevated in the absence of added agonist, but to a lesser degree than the GPIb-normal component. The GPIb-reduced population was again unresponsive to agonist addition.

To further our examination of P-selectin expression, we quantitated the total P-selectin fluorescence, where thrombin-activated fresh platelet fluorescence was assigned a value of 100 (Figure 14). This analysis provides information specific to the degree of degranulation, where thrombin causes a nearly complete degranulation

response. While P-selectin levels were very low on unactivated fresh platelets, ADP/epinephrine addition caused an approximately 50% platelet degranulation. The liquid-preserved platelets have a slightly increased level of P-selectin in the absence of added in vitro agonist, and the response to the 2 agonists used in this study resulted in a similar total degranulation response compared to fresh. The GPIb-normal cryopreserved population had nearly 20% of their total P-selectin exposed on the cell surface prior to agonist addition, and responded to both the ADP/epinephrine and thrombin. The thrombin response by the GPIb-normal population was significantly lower compared to the fresh or liquid-preserved, but the ADP/epinephrine response was not decreased. The GPIb-reduced cryopreserved population also had increased levels of surface P-selectin, but not to the same degree as the GPIb-normal. Again, the GPIb-reduced cryopreserved population was unresponsive to agonist addition.

A representative profile of histograms of the platelet surface markers used in this study are shown in figure 15 comparing 3 day liquid preserved and cryopreserved platelet products. Given that these are log-scale histograms for all parameters, the subtle changes occurring with only 3 days of liquid preservation are not visually apparent, therefore, fresh platelets are not included in this figure. Log forward light scatter, a measure of cell size and refractive index is on the Y axis of all 8 histograms. The appearance of both decreased GPIb and increased factor V binding subpopulations in the cryopreserved product are seen in the far right and left columns. The 2 middle columns represent from left to right, the activated GPIIb-IIIa complex (fibrinogen receptor) and the granule marker P-selectin. The cryopreservation

procedure induced an increase in both these receptors, but distinct subpopulations are not produced. Comparison of multiple surface markers allows correlation of immunological and light scattering signals in a single platelet product. In figure 15 the low GPIb and high factor V subpopulations are both generally lower in log forward light scatter (Y axis). The platelets which express the highest level of activated GPIIb-IIIa and P-selectin by comparison, are those which are higher in log forward light scatter.

## DISCUSSION

It has been determined that compared to 3-4 day 22°C liquid preserved platelets, transfusion of cryopreserved platelets results in a decrease in non-surgical blood loss and transfusion requirements following complex cardiopulmonary bypass procedures.<sup>1</sup> The careful identification and exclusion of surgical bleeding was required to assure that valid conclusions were drawn from this study. Blood loss from a specific site as a result of the surgical procedure itself is referred to as "surgical" as opposed to "non-surgical", a diffuse source of blood loss which is not associated with a specific anatomic site which can be surgically corrected. Since hypothermia and heparin therapy greatly influence platelet function,<sup>5-7</sup> the beneficial effects of platelet transfusion are not fully realized until the patient has been rewarmed and the heparin neutralized with protamine. This is supported by the fact that in the study of Healey et. al.<sup>1</sup> the majority of the reduced blood loss benefit with cryopreserved platelets was observed in the surgical intensive care unit rather than the operating room. The present study was designed to examine liquid- and cryo-preserved platelets products similar to those used in the Healey study, and compare them to fresh platelet preparations.

A consistent observation has been the cryopreserved platelet's ability to produce more thromboxane A<sub>2</sub> (TXA<sub>2</sub>, measured by RIA of thromboxane B<sub>2</sub>, the stable breakdown product of TXA<sub>2</sub>) than either fresh or liquid preserved platelets. TXA<sub>2</sub> is a platelet agonist produced metabolically from arachidonic acid in the platelet membrane which participates in amplification of platelet responses to weak agonists such as ADP

encountered in vivo.<sup>8</sup> In addition, TXA<sub>2</sub> has potent vasoconstrictive properties which would be delivered locally by adherent platelets. TXA<sub>2</sub> may also amplify the vasoconstrictor effects of serotonin. Both the activation and vasoconstrictor effects of TXA<sub>2</sub> are likely to participate in blood loss reduction by increasing platelet recruitment to form an effective platelet plug, and constriction of microcirculatory blood vessels to reduce the flow of blood to the site of injury.<sup>8</sup>

The cryopreserved platelets were much more susceptible to hypotonic stress indicating compromised membrane integrity. Platelet lysis was most likely partially responsible for the incomplete return to baseline light transmittance. Loss of active ion transport across the plasma membrane to maintain cell morphology, and disruption of the platelet cytoskeleton are factors which can contribute to this result.

The slight reduction in plasma pH of the cryopreserved platelets after washing and resuspension in autologous ACD plasma may be due to a combination of effects. Some platelet lysis, which is unavoidable in any freeze-thaw process would liberate the acidic contents of internal platelet organelles, thereby reducing the extracellular pH. In addition, the platelet wash solution used post-thaw is a low pH buffer (pH 5), and any residual wash solution left after expressing the supernatant would have an effect on the final pH, despite the high buffering capacity of the ACD-plasma final resuspension medium.

Another unique feature of cryopreserved platelets is the appearance of a distinct population of platelets with reduced GPIb. The separation from remaining GPIb normal platelets was sufficiently clear to allow separate analysis of these 2 subpopulations.



The GPIb reduced platelets had only ~5% of their original surface GPIb remaining, while the GPIb-normal had >80%. There were approximately equal numbers of each of these subpopulations in the cryopreserved product.

Previous studies have reported that liquid-preserved platelets lose their surface GPIb<sup>9</sup> and this is associated with the appearance of the GPIb fragment glycalicin in the external medium.<sup>10</sup> The reduction of surface GPIb on a subpopulation of platelets observed after cryopreservation may be due to proteolysis by plasmin<sup>11,12</sup>, thrombin<sup>13,14</sup>, neutrophil elastase<sup>15</sup>, or calpains.<sup>16</sup> Membrane vesiculation is unlikely to be the primary cause of GPIb loss in cryopreserved platelets, since forward light scatter reductions consistent with a 30-50% loss of membrane are associated with a >90% loss of surface GPIb. It is likely that compromised membrane integrity after cryopreservation caused an increase in intracellular calcium levels, which is maintained at about 100 nM under normal circumstances. Therefore, the loss of surface GPIb is most likely associated with GPIb proteolysis by endogenous calcium-activated protease or calpain. This hypothesis is supported by the fact that addition of *exogenous* protease inhibitors has been ineffective in preventing GPIb proteolysis in 22°C liquid preservation studies.<sup>16</sup>

In previous studies, there were indications that 2 subpopulations of cryopreserved platelets were present with different agonist responsiveness and expression of surface receptors. For these reasons, in this study we employed a new 3 color flow cytometric technique analyzing the 2 GPIb populations separately to characterize their differences. When surface GPIb fluorescence is plotted against

other signals such as log forward scatter or CD41 using dual parameter histograms, the separation becomes more clear allowing even more efficient individual analysis of subpopulations in the cryopreserved platelet product.

Platelet cryopreservation resulted in a decreased log forward light scatter signal compared to fresh platelets, a finding similar to that observed for liquid preserved platelets.<sup>17</sup> Laser-based automated cell counters have been used to examine the effects of platelet storage at 22°C on the mean platelet volume which utilizes forward light scattering properties.<sup>18</sup> These changes in light scatter are consistent with formation of platelet membrane vesicles or platelet-derived microparticles as has been described in previous studies.<sup>17</sup> Binding of factor V has also been described as an indicator of platelet-derived microparticles generation.<sup>19,20</sup> We combined these properties to define platelet-derived microparticles in this study as having both low forward light scatter and increased factor V binding. Using the multiple immunological parameter detection capabilities of flow cytometry coupled with light scattering signals, we were able to ascertain functional changes which were associated with the decrease in light scattering properties.

It was observed that, in addition to the decreased light scatter and the distinct GPIb normal and reduced subpopulations, there are distinct subpopulations of platelets exhibiting high/low factor V binding in the cryopreserved product. We raised two questions: What are the relative surface-marker relationships between these 2 sets of platelet subpopulations? Are the emerging GPIb-reduced platelets also the events which are exhibiting the increased procoagulant activity? To answer these questions

we used the 3-color flow cytometric technique to separate the GPIb normal and reduced populations, and individually measure their forward light scattering, surface bound factor V, activated GPIIb-IIIa, and P-selectin status with and without agonist addition.

While fresh platelets and those stored for 3 to 4 days at 22°C had minimal surface bound factor V, the cryopreserved platelets were more positive for this coagulation protein. The GPIb-reduced population was completely positive for factor V and exhibited decreased log forward scatter. Therefore, these constituted a homogeneous population classified as platelet-derived microparticles by the definition used in this study. It should be noted that forward light scatter can be influenced by not only particle size, but also the refractive index<sup>21</sup> compared to the surrounding medium, which would be decreased in situations of compromised membrane integrity. The GPIb-normal population were ~15% platelet-derived microparticles without agonist addition, and considerably more responsive to calcium or calcium-containing agonist addition than fresh or liquid-preserved platelets.

Since platelets exhibiting normal forward light scatter are capable of binding considerable levels of factor V, the total factor V binding is a useful measure of the procoagulant activity of the transfusion product. While the cryopreserved GPIb-reduced platelets had high factor V binding, addition of 3 mM CaCl<sub>2</sub> to the anticoagulated product resulted in much more binding. Addition of thrombin and collagen with the calcium did not cause any further increase in factor V binding. The GPIb-normal population also exhibited increased factor V binding compared to fresh or

liquid preserved platelets under these conditions. Given the fact that the GPIb-normal subpopulation represents more than 50% of the platelet-specific events found in the cryopreserved product, the surface factor V activity of this subpopulation, while lower than the GPIb-reduced subpopulation represents a significant source of procoagulant activity. Addition of calcium elicited the binding of factor V to a unique subpopulation of the GPIb-normal platelets. This group had very high factor V binding, but no decrease in forward light scatter. In vitro agonist addition to fresh platelets is associated with a responsive population which has lower forward light scatter, as observed in this study and others.<sup>19,20</sup>

The exposure of the fibrinogen binding site on the GPIIb-IIIa complex is an important indicator of platelet function since fibrinogen binding and subsequent inter-platelet crosslinking is the predominate mechanism by which platelets aggregate in response to physiologic agonists.<sup>22</sup> In vivo, the aggregation response is primarily responsible for physical platelet recruitment to the site of thrombus formation. GPIIb-IIIa is a calcium dependent complex which undergoes conformation changes upon stimulation with agonists in vivo and in vitro.<sup>23</sup> Without continuous stimulation, the complex will revert to the unactivated form, as has been observed in a model of thrombus formation under flow conditions.<sup>24</sup> The reversible functional state of this receptor therefore allows us to determine the current activation state but not the occurrence of stimulation at a previous point in time. For these reasons, response to in vitro agonist addition may be a more valuable measurement of function than the "resting" level taken at the time of assay. In fact, due to the calcium dependent nature

of this complex, even the presence of divalent cationic chelators such as EDTA and citrate can elicit changes in the receptor similar to physiologic ligand binding, which is relevant when studying an anticoagulated blood product.

The GPIb-normal cryopreserved platelets retained their ability to conform their GPIIb-IIIa to the activated state, while the GPIb-reduced population did not. While the GPIb-normal population did expose less (~65%) activated GPIIb-IIIa than either fresh or liquid stored platelets, there are ~50,000 copies of GPIIb-IIIa on the platelet surface. This degree of exposure is more than sufficient to promote aggregation of platelets. In this study, the aggregation response to ADP plus arachidonic acid of cryopreserved platelets was reduced. The decreased response to this agonist combination was probably partially compensated for by the increased production of thromboxane by these same platelets. While the activated GPIIb-IIIa response of liquid preserved platelets was as robust as the fresh, the decreased production of thromboxane, which serves as a platelet activation amplification mechanism, was likely to be responsible for the decreased aggregation to ADP plus arachadonic acid compared to fresh platelets.

P-selectin is an  $\alpha$ -granule protein which is surface exposed with platelet degranulation and mediates the binding of activated platelets to the surface of granulocytes and monocytes both in vivo and in vitro.<sup>25,26</sup> As previously reported<sup>27,28</sup> 22°C liquid-storage of platelets results in an increased level of surface P-selectin. In the present study, 3-4 day liquid preservation resulted in an increased surface P-selectin level and a slightly reduced percentage of platelets responding to in vitro agonist addition. The GPIb-normal platelets had considerably higher surface P-

selectin than either fresh or liquid preserved and a diminished response to thrombin but not a combination of ADP and epinephrine. The GPIb-reduced cryopreserved platelets had elevated P-selectin, but not to the degree of the GPIb-normal population, and were completely unresponsive to agonist addition.

The following is a possible sequence of events which would explain these findings: The increased procoagulant activity we observed by surface bound factor V would ultimately lead to thrombin generation and cleavage of a portion of the N terminus of the seven-transmembrane domain thrombin receptor.<sup>29</sup> The GPIb-reduced platelets, which have probably sustained greater freeze-thaw damage would have an attenuated response to the generated thrombin due to breakdown of various signal transduction pathways. In addition, they are likely to have released activating substances (ADP and serotonin from dense granules for example) at this time when they are less able to respond to exogenous agonist. In contrast, the accompanying GPIb-normal platelets, sustaining minimal freeze-thaw damage would retain their ability to respond to these agonists. These platelets, having been previously stimulated during the thawing and washing process and having lost a portion of their original thrombin receptors, would be refractory to later activation, particularly by thrombin.

While the presence of surface P-selectin indicates platelet activation has occurred, this does not necessarily correlate with a loss of cell survival and function.<sup>30</sup> This is supported by the fact that platelet survival and function after human transfusion of cryopreserved platelets,<sup>1</sup> which are relatively high in surface P-selectin resulted in good survival of those platelets initially recovered in vivo. In circumstances where

platelets must act to provide procoagulant activity and vasoactive metabolites (e.g. thromboxane) some consumption of circulating platelets must be expected. Platelet survival alone cannot be used as a measure of the functional ability of the transfusion product to improve the clinical outcome, particularly in cases of severe trauma.

Platelet function assays of the types described in this study will allow detailed examination of various collection techniques and platelet-storage modalities. This information will enable researchers, and ultimately the clinical laboratory technician to modify current procedures to improve the hemostatic effectiveness of the growing number of available platelet products which can be administered to the cardio-pulmonary bypass patient, and in other clinical settings.

## REFERENCES

1. Healey NA, Khuri S, Valeri CR, MacGregor H, Doursounian M, Zolkewitz MA, Patel M, Birjiniuk V, Khait I: Platelets stored at -80°C for 1.3 years: A new option for post-cardiopulmonary bypass transfusion therapy. *Circulation* 92:I-764, 1995 (abstr.)
2. Murphy S, Rebullia P, Bertolini F, Holme S, Moroff G, Snyder E, Stromberg R: In vitro assessment of the quality of stored platelet concentrates. *Trans Med Reviews* VIII:29, 1994
3. Melaragno AJ, Abdu WA, Katchis RJ, Vecchione JJ, Valeri CR: Cryopreservation of platelets isolated with the IBM 2992 blood cell separator: a rapid and simplified approach. *Vox Sang* 43:321, 1982
4. Michelson AD, Ellis PA, Barnard MR, Matic GB, Viles AF, Kestin AS: Downregulation of the platelet surface glycoprotein Ib-IX complex in whole blood stimulated by thrombin, ADP or an in vivo wound. *Blood* 77:770, 1991
5. Michelson AD, MacGregor H, Barnard MR, Kestin AS, Rohrer MJ, Valeri CR: Reversible inhibition of human platelet activation by hypothermia in vivo and in vitro. *Thromb Haemost* 71:633, 1994



6. Kestin AS, Valeri CR, Khuri SF, Loscalzo J, Ellis PA, MacGregor H, Birjiniuk V, Ouimet H, Pasche B, Nelson MJ, Benoit SE, Rodino LJ, Barnard MR, Michelson AD: The platelet function defect of cardiopulmonary bypass. *Blood* 82:107, 1993
7. Upchurch GR, Valeri CR, Khuri SF, Rohrer MJ, Welch GN, MacGregor H, Ragno G, Francis S, Rodino LJ, Michelson AD, Loscalzo J: Effect of heparin on fibrinolytic activity and platelet function in vivo. *Am J Physiol* 271:H528, 1996
8. Marcus AJ: Platelet eicosanoid metabolism, in Colman RW, Hirsh J, Marder VJ, Salzman EW (eds): Hemostasis and thrombosis. Basic principles and clinical practice, Philadelphia, Lippincott, 1987, p 676
9. Michelson AD, Benoit SE, Barnard MR, MacGregor H, Valeri CR: Blood bank storage of platelet concentrates results in a loss of platelet surface GPIb (CD42b) and GPV (CD42d) but not GPIX (CD42a). in Schlossman SF, Boumsell L, Gilks W, Harlan JM, Kishimoto T, Morimoto C, Ritz J, Shaw S, Silverstein RL, Springer TA, Tedder TF, Todd RF (eds): Leukocyte Typing V, Oxford, Oxford University Press, 1995, p 1331
10. Michelson AD, Adelman B, Barnard MR, Carroll E, Handin RI: Platelet storage results in a redistribution of glycoprotein Ib molecules. Evidence for a large intraplatelet pool of glycoprotein Ib. *J Clin Invest* 81:1734, 1988

11. Adelman B, Michelson AD, Handin RI, Ault KA: Evaluation of platelet glycoprotein Ib by fluorescence-activated flow cytometry. *Blood* 66:423, 1985
12. Adelman B, Michelson AD, Greenberg J, Handin RI: Proteolysis of platelet glycoprotein Ib by plasmin is facilitated by plasmin lysine-binding regions. *Blood* 68:1280, 1986
13. Michelson AD, Barnard MR: Thrombin-induced changes in platelet membrane glycoproteins Ib, IX, and IIb-IIIa complex. *Blood* 70:1673, 1987
14. George JN, Pickett EB, Saucerman S, McEver RP, Kunicki TJ, Kieffer N, Newman PJ: Platelet surface glycoproteins. Studies on resting and activated platelets and platelet membrane microparticles in normal subjects, and observations in patients during adult respiratory distress syndrome and cardiac surgery. *J Clin Invest* 78:340, 1986
15. Sloand EM, Klein HG: Effect of white cells on platelets during storage. *Transfusion* 30:333, 1990
16. Bode AP, Knupp CL, Miller DT: Effect of platelet activation inhibitors on the loss of glycoprotein Ib during storage of platelet concentrates. *J Lab Clin Med* 115:669, 1990

17. Bode AP, Orton SM, Frye MJ, Udis BJ: Vesiculation of platelets during in vitro aging. *Blood* 77:887, 1991
18. Seghatchian J, Krailadsiri P: The platelet storage lesion. *Trans Med Reviews* 11:130, 1997
19. Sims PJ, Faioni EM, Wiedmer T, Shattil SJ: Complement proteins C5b-9 cause release of membrane vesicles from the platelet surface that are enriched in the membrane receptor for coagulation factor Va and express prothrombinase activity. *J Biol Chem* 263:18205, 1988
20. Abrams CS, Ellison N, Budzynski AZ, Shattil SJ: Direct detection of activated platelets and platelet-derived microparticles in humans. *Blood* 75:128, 1990
21. Udis BJ, Hickerson D, Bode AP: Vesiculation versus light scatter shifts in activated platelets. *Cytometry* 54, 1994 (abstr.)
22. Phillips DR, Charo IF, Parise LV, Fitzgerald LA: The platelet membrane glycoprotein IIb-IIIa complex. *Blood* 71:831, 1988
23. Shattil SJ, Hoxie JA, Cunningham M, Brass LF: Changes in the platelet membrane glycoprotein IIb-IIIa complex during platelet activation. *J Biol Chem* 260:11107, 1985

24. Heynen HF, Lozano M, de Groot PG, Nieuwenhuis HK, Sixma JJ: Absence of ligands to glycoprotein IIb-IIIa on the exposed surface of a thrombus may limit thrombus growth in flowing blood. *J Clin Invest* 94:1098, 1994
25. Larsen E, Celi A, Gilbert GE, Furie BC, Erban JK, Bonfanti R, Wagner DD, Furie B: PADGEM protein: A receptor that mediates the interaction of activated platelets with neutrophils and monocytes. *Cell* 59:305, 1989
26. Hamburger SA, McEver RP: GMP-140 mediates adhesion of stimulated platelets to neutrophils. *Blood* 75:550, 1990
27. Rinder HM, Snyder EL, Bonan JL, Napychank PA, Malkus H, Smith BR: Activation in stored platelet concentrates: correlation between membrane expression of P-selectin, glycoprotein IIb/IIIa, and beta-thromboglobulin release. *Transfusion* 33:25, 1993
28. Rinder HM, Murphy M, Mitchell JG, Stocks J, Ault KA, Hillman RS: Progressive platelet activation with storage: evidence for shortened survival of activated platelets after transfusion. *Transfusion* 31:409, 1991

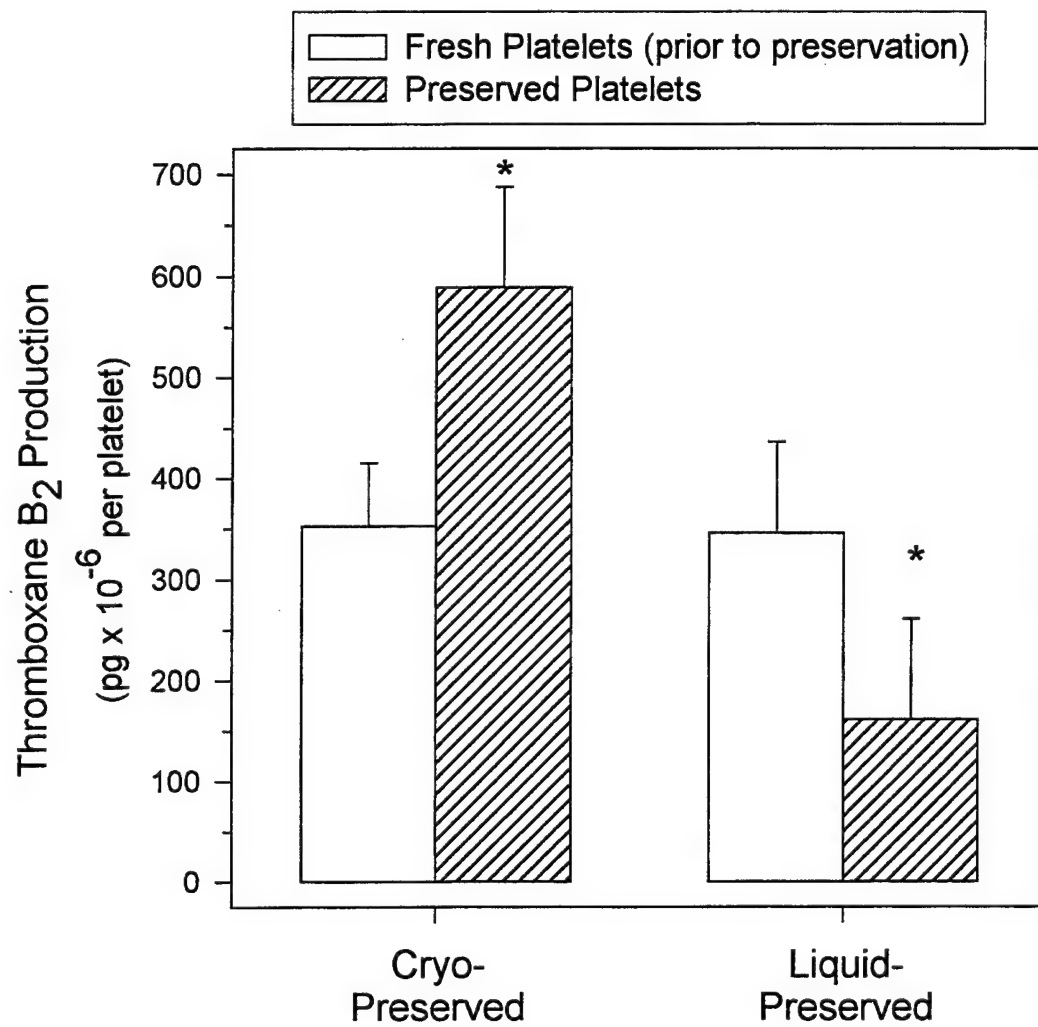
29. Vu TH, Hung DT, Wheaton VI, Coughlin SR: Molecular cloning of a functional thrombin receptor reveals a novel proteolytic mechanism of receptor activation. *Cell* 64:1057, 1991
30. Michelson AD, Barnard MR, Hechtman HB, MacGregor H, Connolly RJ, Loscalzo J, Valeri CR: In vivo tracking of platelets: circulating degranulated platelets rapidly lose surface P-selectin but continue to circulate and function. *Proc Natl Acad Sci USA* 93:11877, 1996

**FIGURE 1** Effect of preservation on platelet production of thromboxane

Effect of preservation on platelet production of thromboxane B<sub>2</sub> in response to a combination of 0.5 mg/mL arachadonic acid and 0.2 mM adenosine diphosphate.

Cryopreserved platelets (n=12) produce significantly more thromboxane B<sub>2</sub> than fresh platelets (n=12). Forty eight hour 22°C liquid storage (n=5) resulted in significantly less production of thromboxane B<sub>2</sub> after stimulation compared to fresh platelets (n=5). Data are mean ± SEM, \* indicates p-value < 0.05 compared to fresh.

FIGURE 1

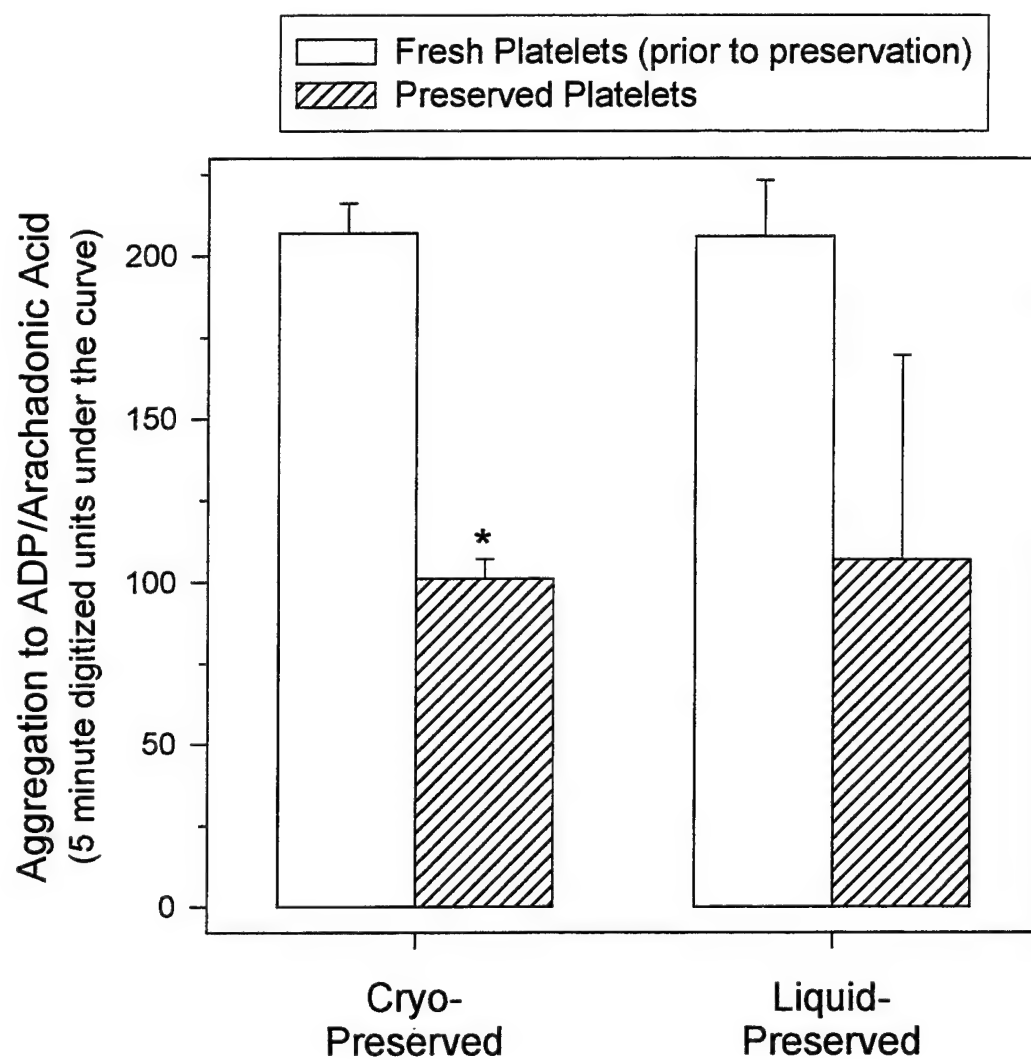


**FIGURE 2** Effect of preservation on platelet aggregation

The effect of preservation on platelet aggregation in response to a combination of 0.5 mg/mL arachadonic acid and 0.2 mM adenosine diphosphate. Total aggregation is reported as the digitized area under the tracing of increased light transmittance after agonist addition. Cryopreserved platelets aggregate significantly less after activation by the combination of arachadonic acid and adenosine diphosphate than fresh platelets (n=12). Forty eight hour 22°C liquid storage also resulted in less aggregation to this agonist combination compared to fresh platelet values (n=5), but with greater data variability. Data are mean  $\pm$  SEM, \* indicates p-value < 0.05 compared to fresh.



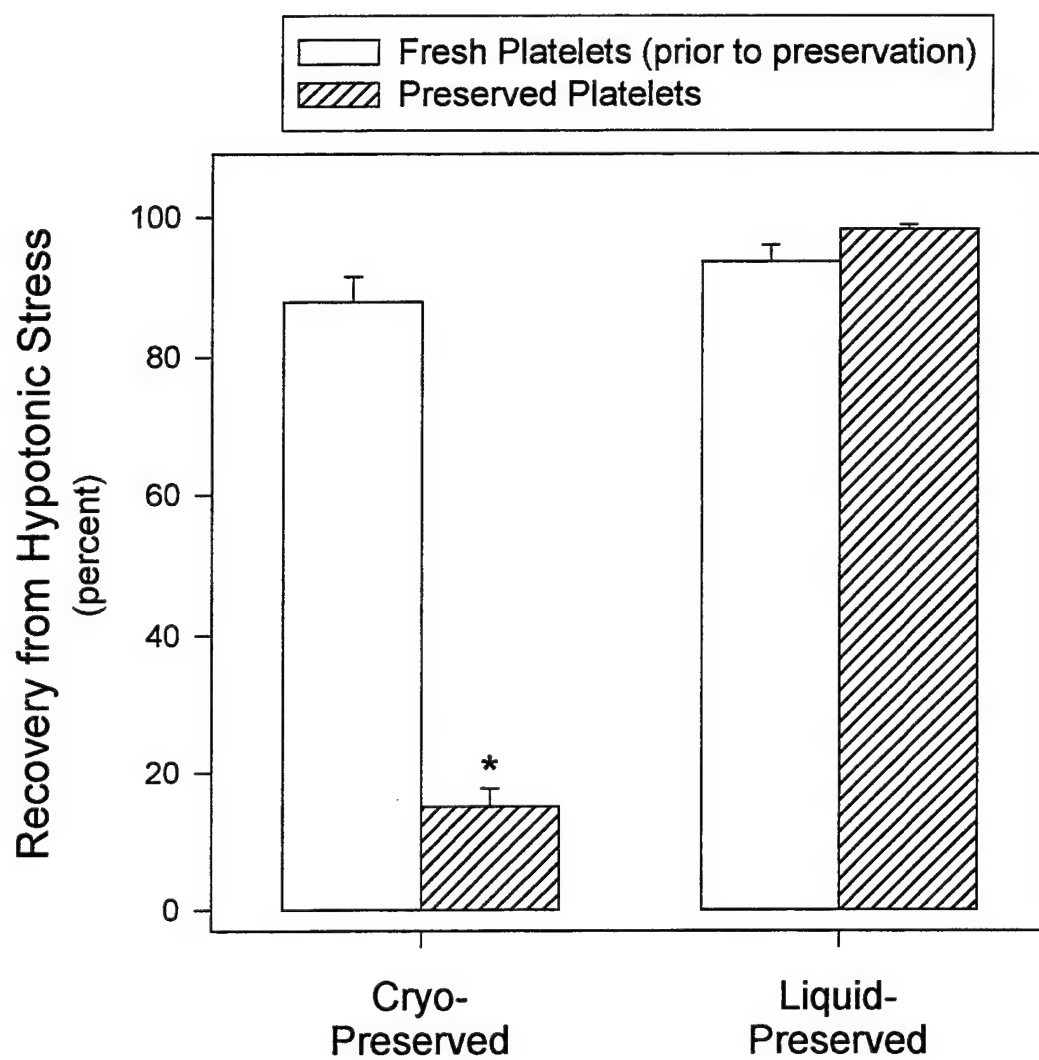
FIGURE 2



**FIGURE 3** Effect of platelet preservation on recovery from hypotonic stress

The effect of platelet preservation on plasma membrane integrity as measured by recovery from hypotonic stress. Cryopreserved platelets were significantly more sensitive to hypotonic stress than the fresh plateletpheresis product (n=9). Forty eight hour 22°C liquid stored platelets were able to recover from hypotonic stress as well as fresh platelets (n=5). Data are mean  $\pm$  SEM, \* indicates p-value < 0.05 compared to fresh.

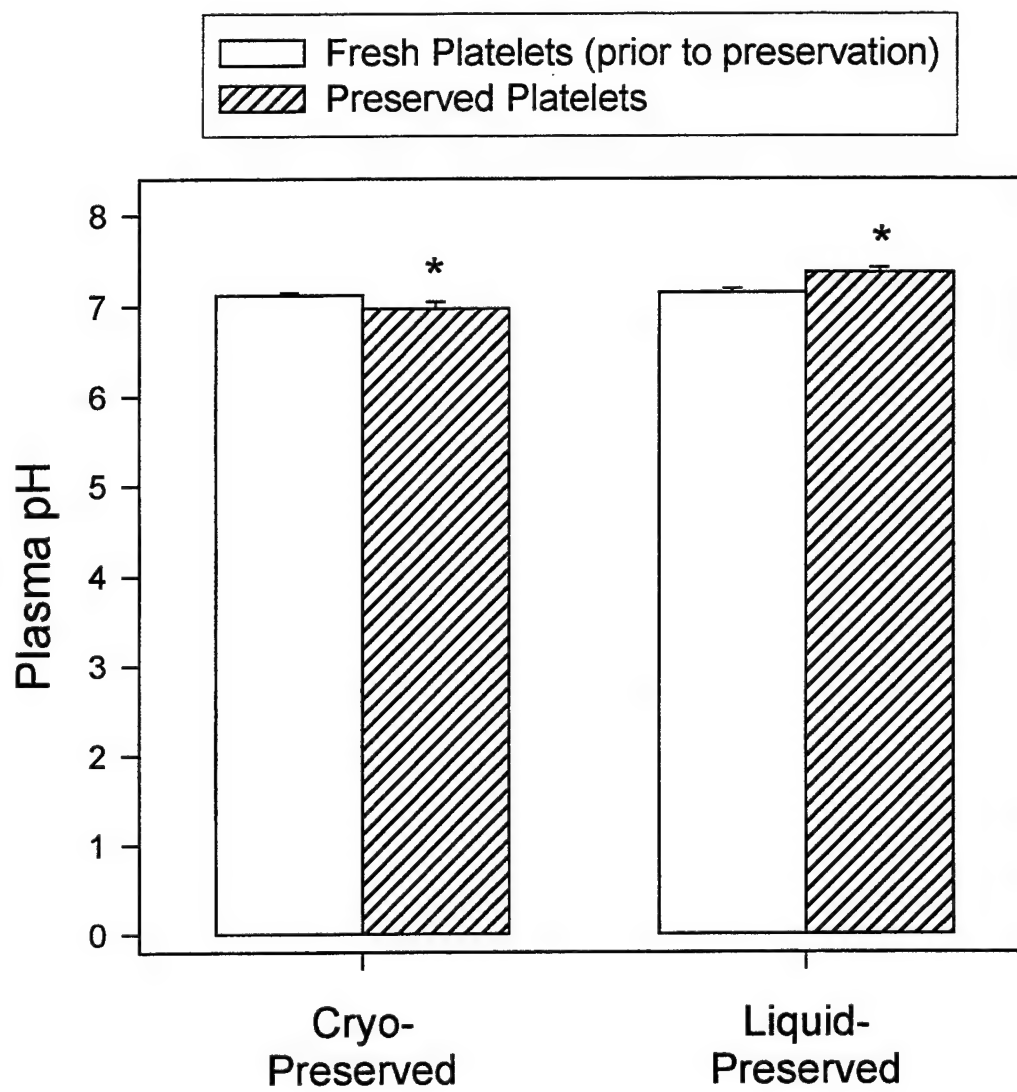
FIGURE 3



**FIGURE 4** Effect of the platelet cryopreservation procedure and 22°C liquid storage on plasma pH

The effect of the platelet cryopreservation procedure and 22°C liquid storage on plasma pH. The pH of thawed-washed cryopreserved platelets was slightly lower than fresh platelets after resuspension in autologous fresh-frozen plasma (n=12). The pH of 48 hour 22°C liquid stored platelets was slightly higher than fresh platelets (n=5). Data are mean  $\pm$  SEM, \* indicates p-value < 0.05 compared to fresh.

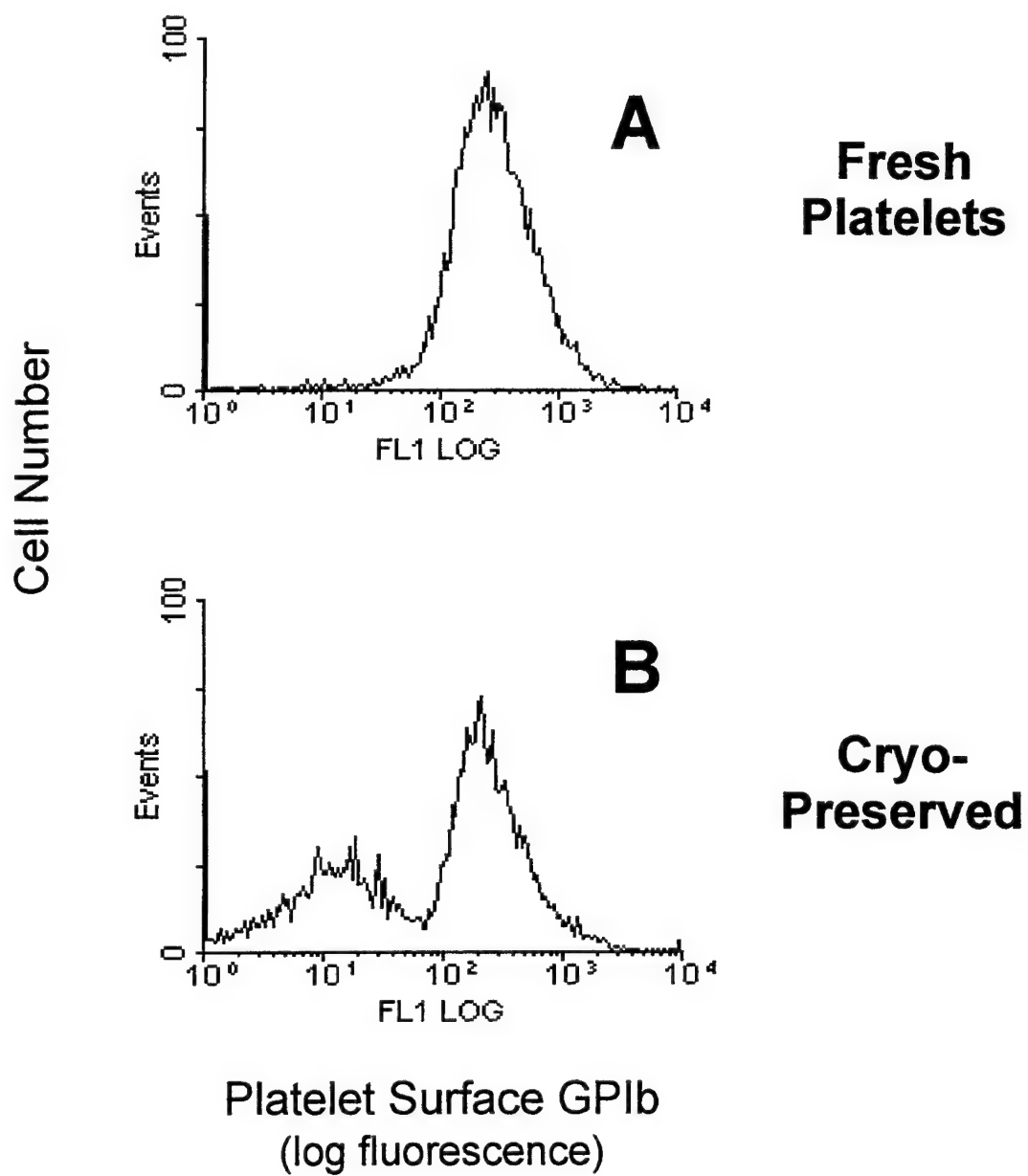
FIGURE 4



**FIGURE 5** Histograms illustrating the effect of cryopreservation on the platelet surface expression of GPIb

Representative histograms illustrating the effect of cryopreservation on the platelet surface expression of GPIb. Fresh platelets (panel A) and cryopreserved platelets (panel B) were incubated with FITC-conjugated antibody 6D1 and analyzed by flow cytometry. Platelets were identified with anti-CD41 antibody. Cryopreservation resulted in the appearance of a GPIb-reduced (left side of panel B) subpopulation which was distinctly separable from the GPIb-normal subpopulation (left side of panel B).

FIGURE 5

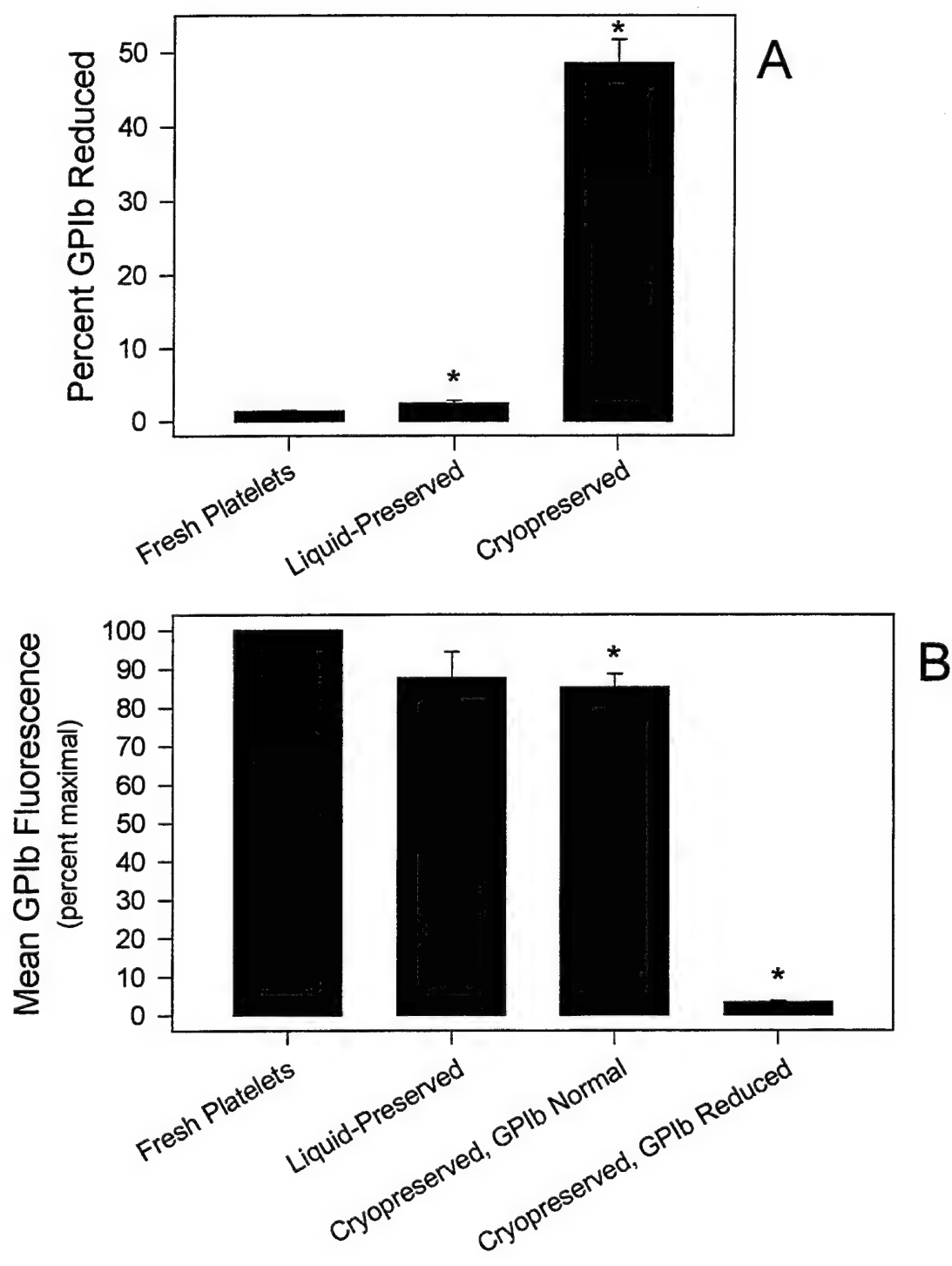


**FIGURE 6** The surface expression of GPIb on fresh, liquid preserved and cryopreserved platelets

The surface expression of GPIb on fresh, 3/4 day liquid preserved and cryopreserved platelets. Each were incubated with FITC-conjugated antibody 6D1 and analyzed by flow cytometry. Platelets were identified with anti-CD41 antibody. Panel A reports the percent of the total population which were GPIb-reduced. Fresh (n=6) and liquid-preserved (n=5) have negligible GPIb-reduced platelet components, while cryopreserved platelets (n=6) contained  $48.6 \pm 3.7\%$  (mean  $\pm$  SE) GPIb-reduced platelets. Panel B shows the quantitative differences in surface expression for each of these preparations. Liquid preservation resulted in a slight reduction of GPIb on day 3/4 of storage. The 2 subpopulations seen in the cryopreserved preparation consisted of GPIb- normal and GPIb-reduced platelets. \* indicates p-value < 0.05 compared to fresh.

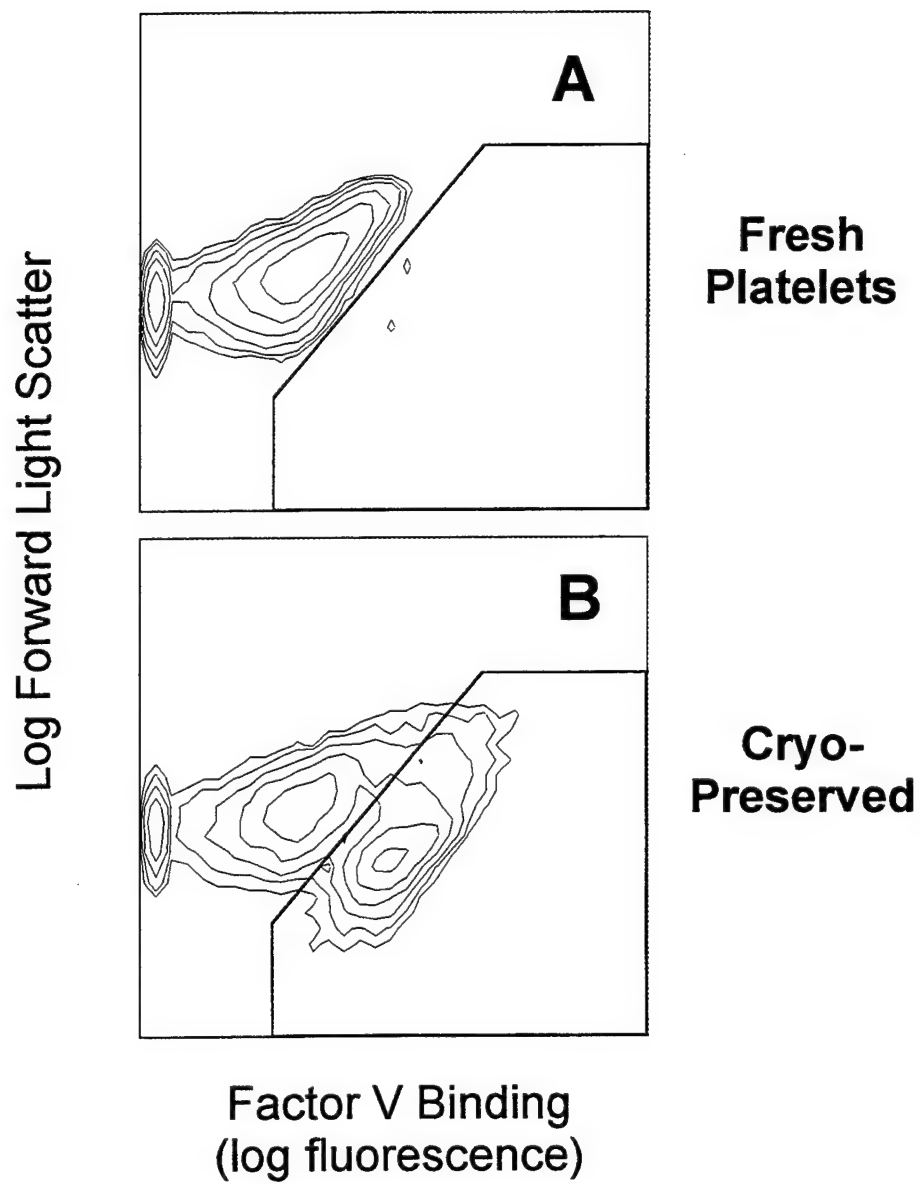


FIGURE 6



**FIGURE 7** Histograms illustrating the analysis of platelet derived microparticles in fresh and cryopreserved platelets

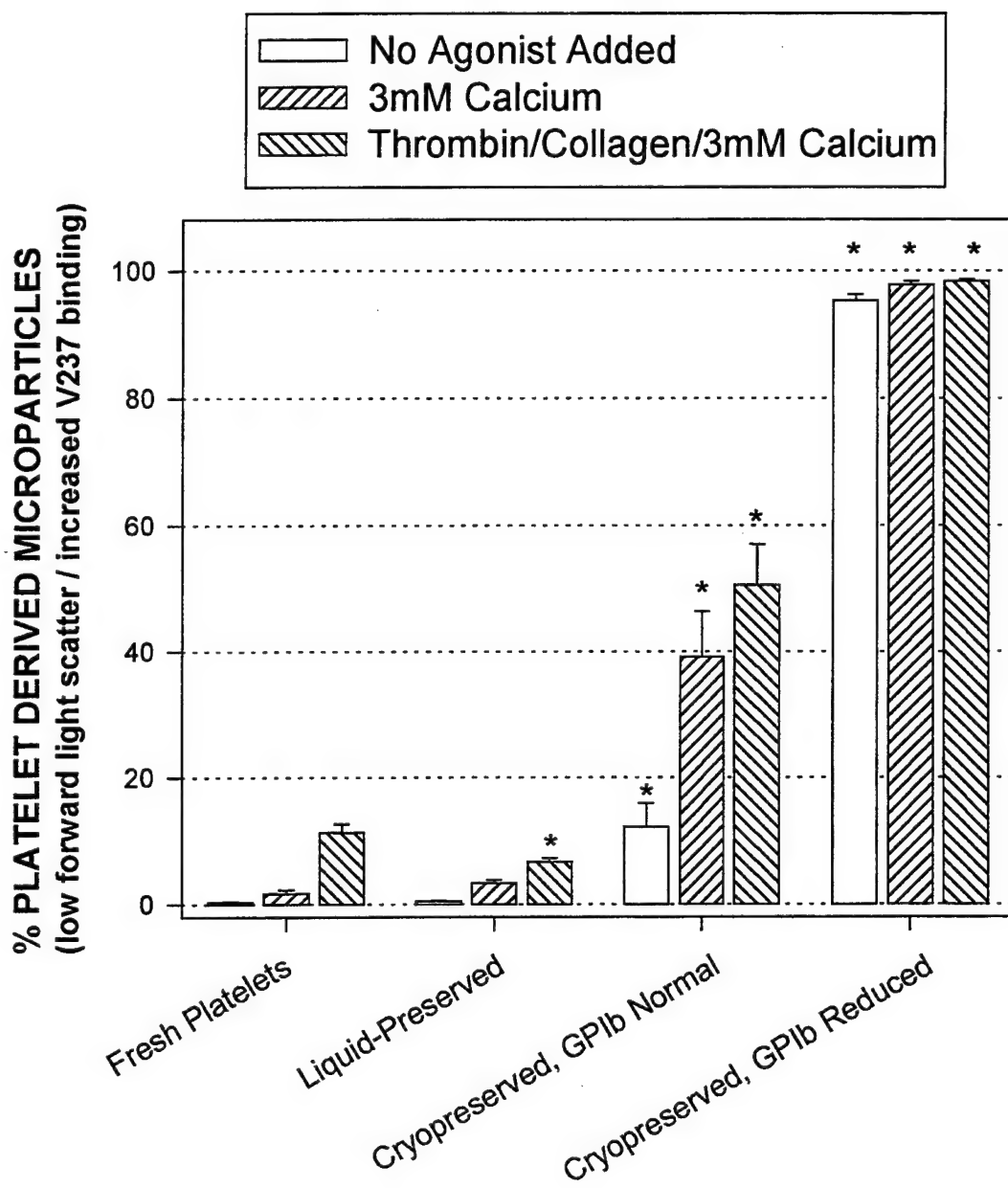
Representative 2 parameter histograms illustrating the method of analysis of platelet derived microparticles in fresh and cryopreserved platelets. A region was established which included events identified with anti-CD41 antibody which were relatively low in log forward light scatter and high in factor V binding. In the absence of added agonist, fresh platelets (panel A) contain very few platelet-derived microparticles which fall in this region, whereas cryopreserved platelets (panel B) contain a large proportion of factor V binding (pro-coagulant) platelet-derived microparticles.

FIGURE 7

**FIGURE 8** Effect of preservation on production of platelet derived microparticles

The effect of 22°C liquid preservation, cryopreservation and addition of in vitro agonists on production of platelet-derived microparticles. To platelets diluted in buffer containing GPRP (to inhibit fibrin clot formation) was added either buffer (no agonist), 3 mM  $\text{CaCl}_2$  (a physiologic concentration), or a combination of thrombin (2 units/mL), collagen (20  $\mu\text{g/mL}$ ) and 3 mM  $\text{CaCl}_2$ . Platelets were identified with anti-CD41 antibody. Liquid-preserved platelets (n=5) produced slightly less platelet-derived microparticles than fresh platelets (n=5) in response to the thrombin/collagen/calcium combination. The 2 GPIb-normal and -reduced subgroups of the cryopreserved platelets (n=4) had elevated platelet-derived microparticles at all agonist doses although there were quantitative differences between the 2 populations. The GPIb-reduced population were composed of virtually all platelet-derived microparticles. The GPIb-normal population while containing increased numbers of platelet-derived microparticles without added agonist, were more responsive to calcium and agonist additions than the fresh platelets. Data are mean  $\pm$  SEM, \* indicates p-value < 0.05 compared to fresh.

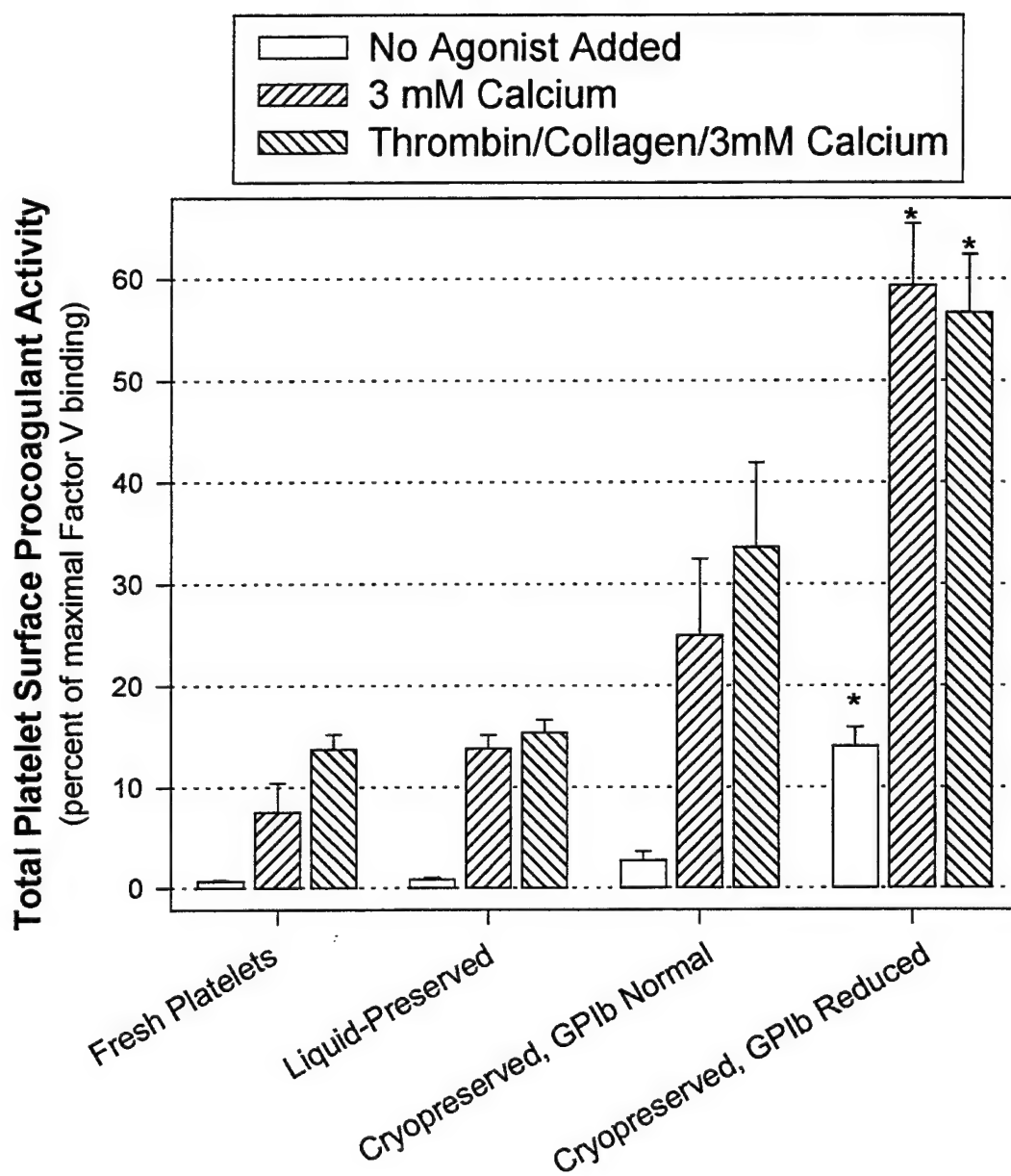
FIGURE 8



**FIGURE 9** Effect preservation on total platelet procoagulant activity

The effect of 22°C liquid preservation, cryopreservation and addition of in vitro agonists on total platelet procoagulant activity. Binding of anti factor V antibody was quantitated in samples to which either buffer (no agonist), 3 mM  $\text{CaCl}_2$  (a physiologic concentration), or a combination of thrombin (2 units/mL), collagen (20  $\mu\text{g/mL}$ ) and 3 mM  $\text{CaCl}_2$  was added. Platelets were identified with anti-CD41 antibody. Liquid-preserved platelet (n=5) procoagulant activity was similar to that of fresh platelets (n=5) at all agonist doses. The GPIb-normal and -reduced subgroups of the cryopreserved platelets (n=4) had elevated activity at all agonist doses with significant quantitative differences between the 2 populations. The GPIb-reduced population had high activity without added agonist, which increased in the presence of calcium. The GPIb-normal population had less activity than the GPIb-reduced population, but was more responsive to calcium and thrombin/collagen/calcium addition than the fresh platelets. Data are mean  $\pm$  SEM, \* indicates p-value < 0.05 compared to fresh.

FIGURE 9

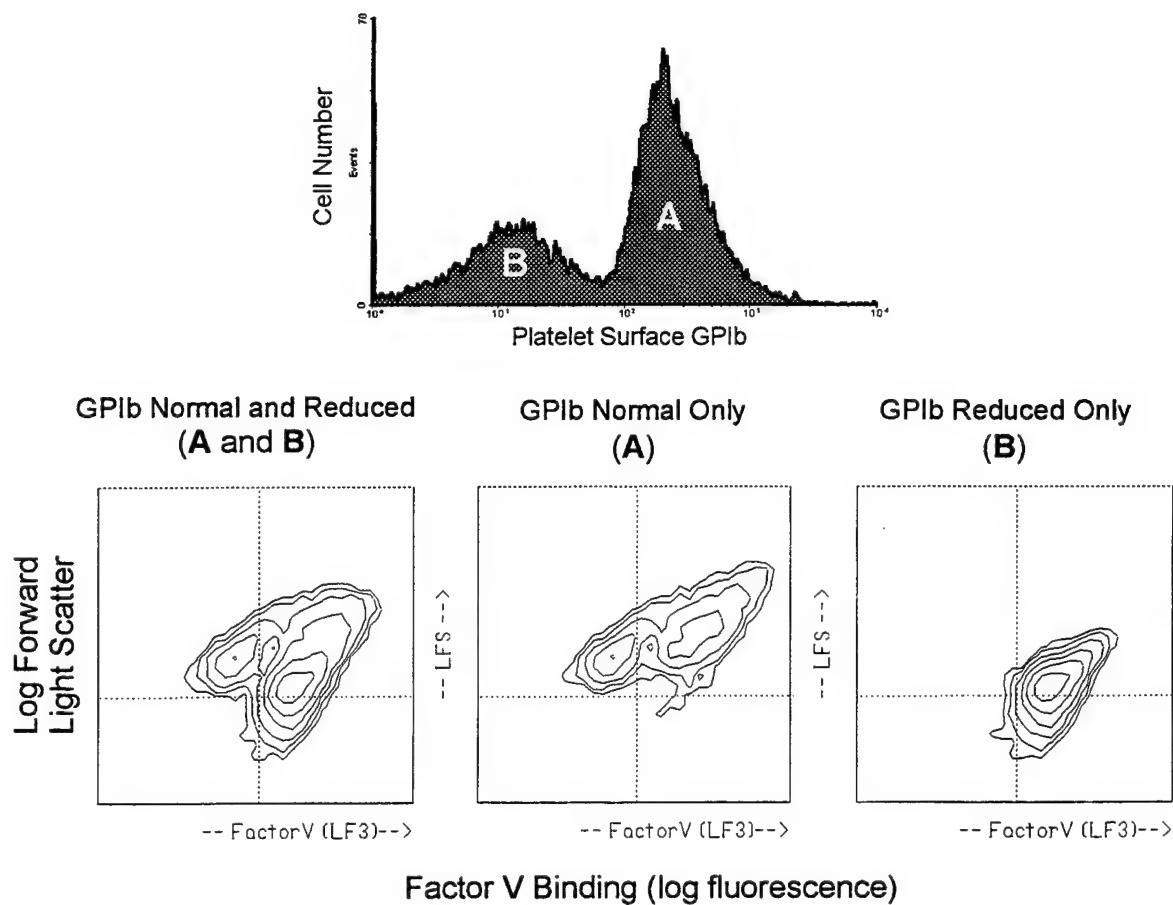


**FIGURE 10** Cryopreserved platelet histograms comparing GPIb normal and reduced subpopulations binding of factor V

Cryopreserved platelet histograms comparing GPIb-normal (A) and GPIb-reduced (B) binding of factor V after addition of 3 mM  $\text{CaCl}_2$ . Using 3 color flow cytometry, platelets were identified with anti-CD41 antibody, GPIb was measured by antibody 6D1 and bound factor V was measured by antibody V237. The bottom left panel shows factor V binding (X axis) vs. forward light scatter (relative size, Y axis) of all CD41 positive events (both A and B peaks of the top histogram). When only the GPIb normal (peak A, top histogram) is plotted, 2 distinct populations with variable factor V binding are seen, each with relatively high forward light scatter (bottom middle panel). When only the GPIb reduced (peak B, top histogram) is plotted, a single population of high factor V binding is seen (bottom left panel).



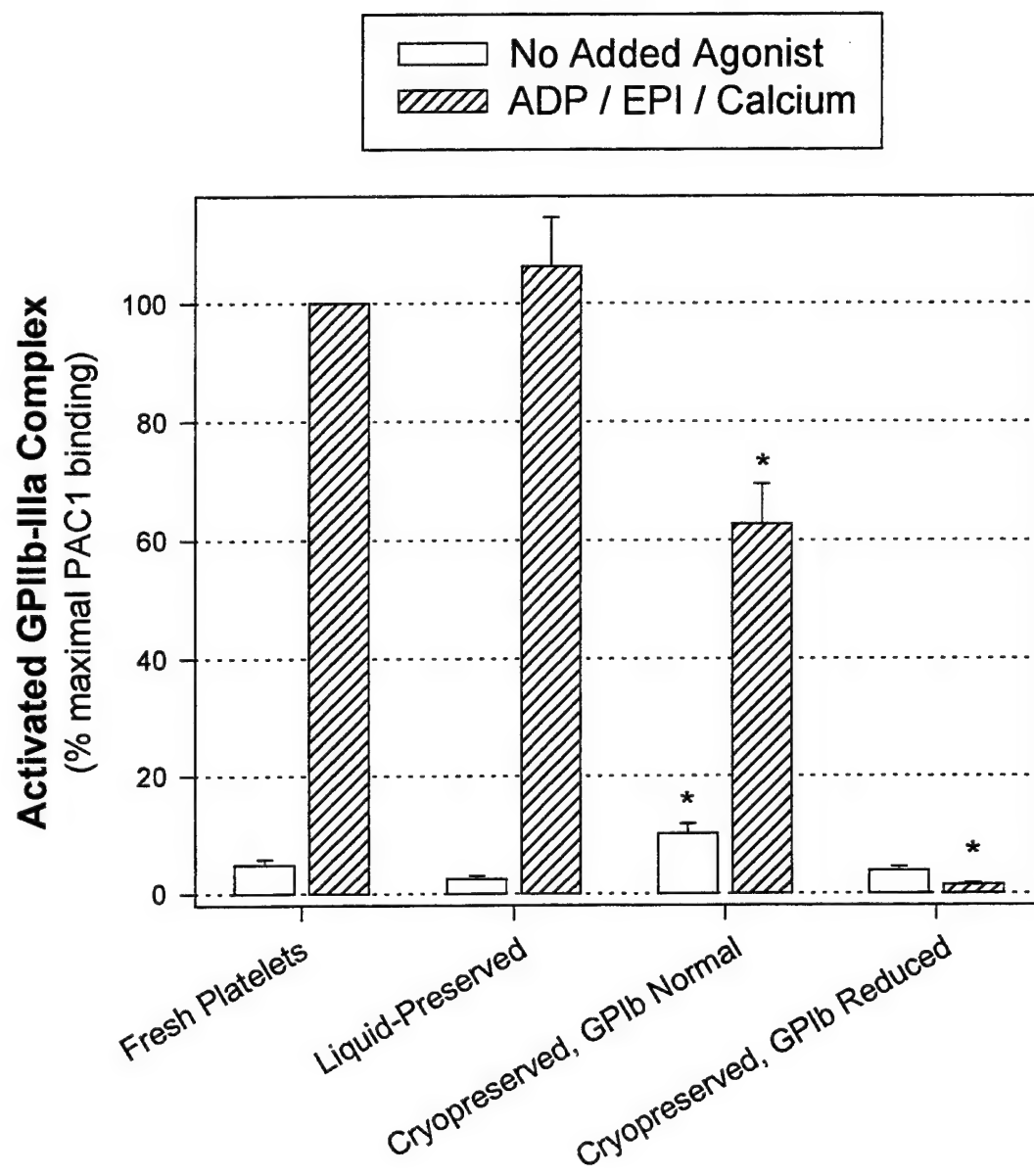
FIGURE 10



**FIGURE 11** Effect of platelet preservation on the exposure of the fibrinogen binding site on the GPIIb-IIIa complex

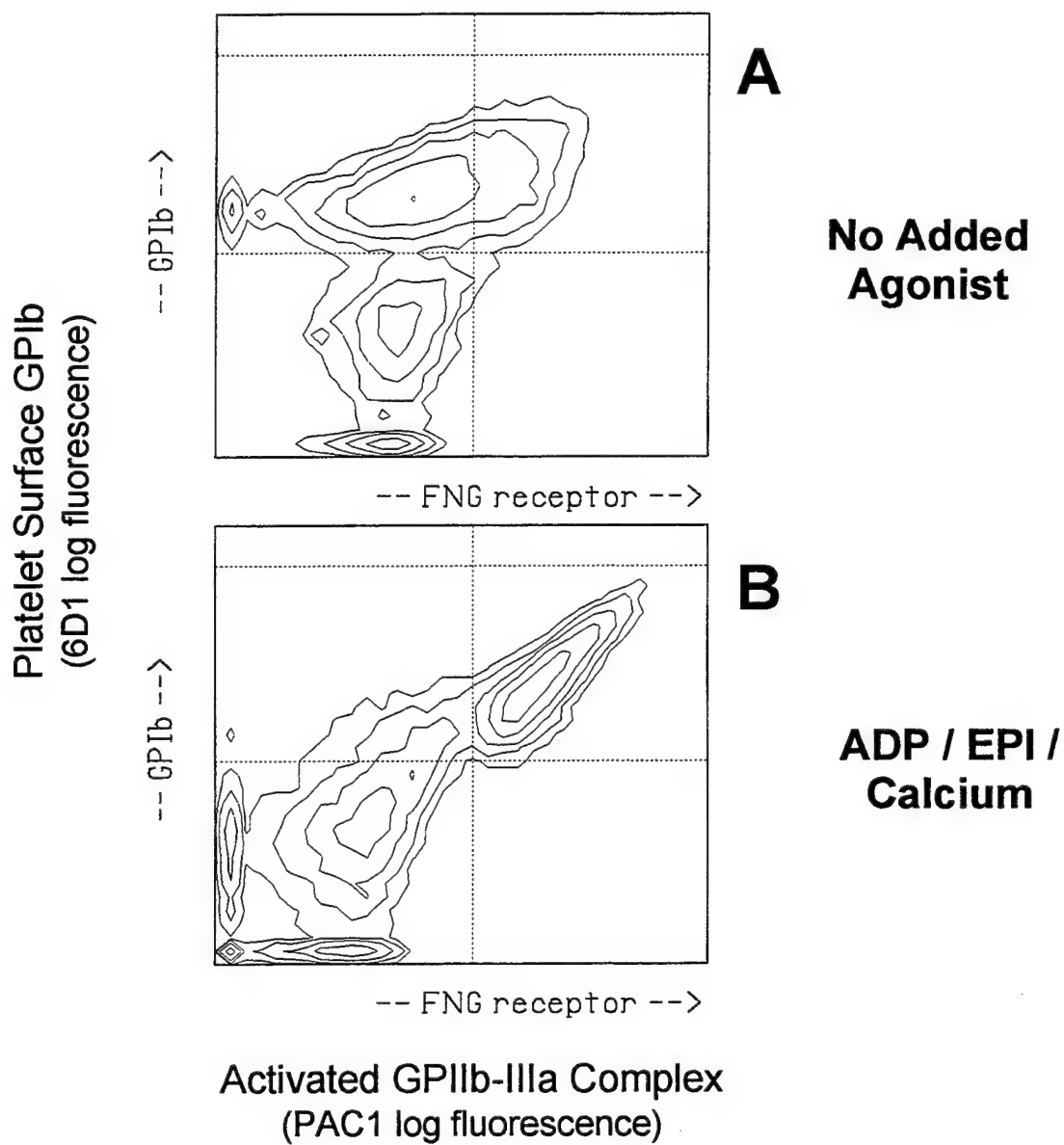
Effect of platelet preservation on the exposure of the fibrinogen binding site on the GPIIb-IIIa complex with and without added agonist. Platelets were identified with anti-CD41 antibody. Day 3/4 22°C liquid storage (n=5) did not cause spontaneous activation of GPIIb-IIIa, and did not effect the response to a combination of 20  $\mu$ M ADP / 20  $\mu$ M epinephrine / 3 mM CaCl<sub>2</sub> compared to fresh platelets (n=5). The cryopreserved (n=8) GPIb-normal subpopulation had slightly more activated GPIIb-IIIa without agonist and was still able respond to the combination of agonists, although to a lesser extent than fresh platelets. The cryopreserved GPIb-reduced subpopulation had less activated GPIIb-IIIa without agonist compared to the GPIb-normal cryopreserved platelet component, and was unable to respond to agonist. Data are mean  $\pm$  SEM, \* indicates p-value < 0.05 compared to fresh.

FIGURE 11



**FIGURE 12** Contour plots of cryopreserved platelets illustrating the GPIIb-IIIa responsiveness of the GPIb subpopulations

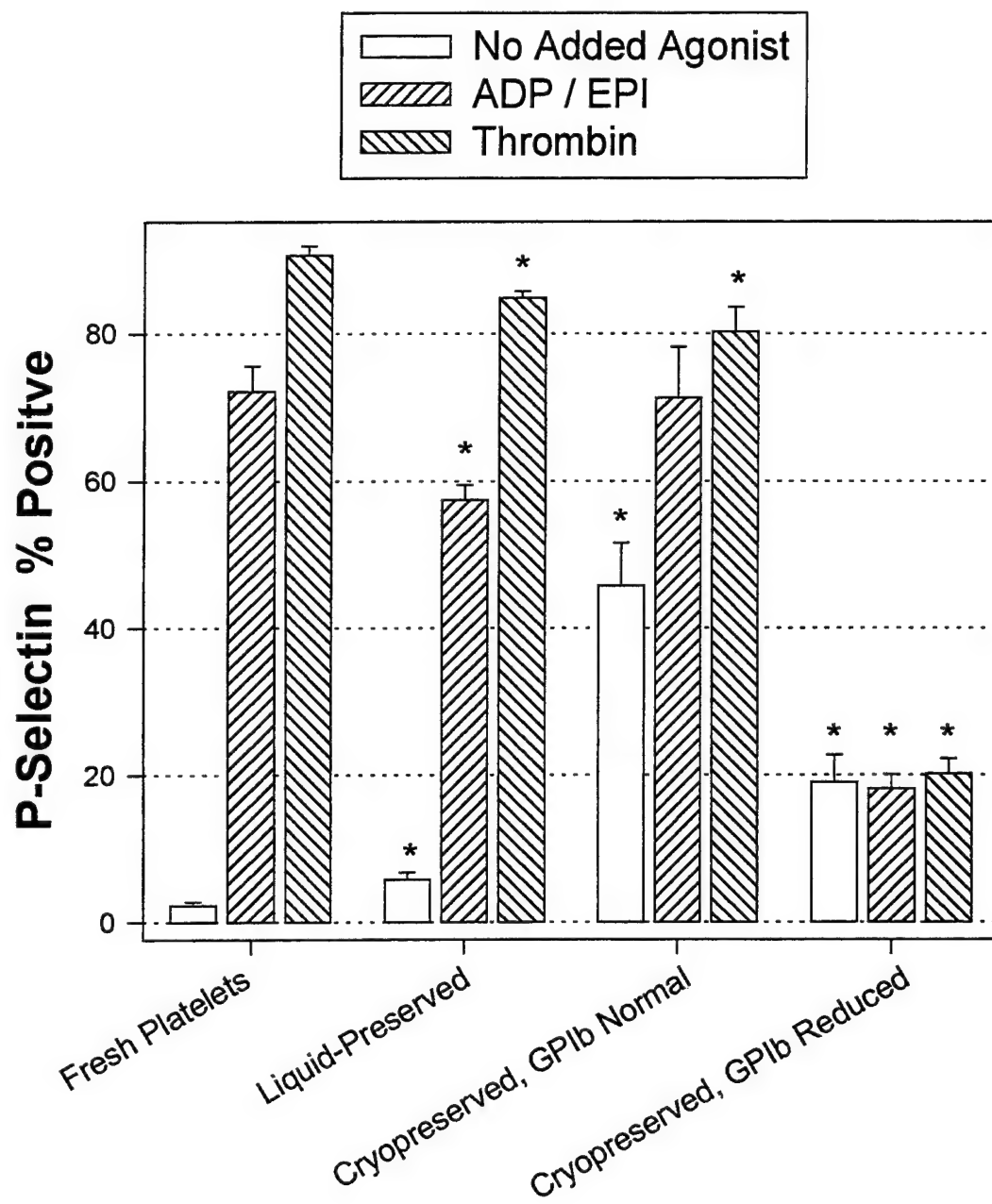
Representative contour plots of cryopreserved platelets illustrating the responsiveness to agonist of the GPIb-normal and -reduced subpopulations. Platelets were identified with anti-CD41 antibody. Binding of PAC1 directed against the activated GPIIb-IIIa complex is on the X axis and binding of 6D1 directed against surface GPIb is on the Y axis. Panel A shows the relatively higher background binding of PAC1 on the GPIb-normal (upper) population. Panel B shows addition of a combination of 20  $\mu$ M ADP / 20  $\mu$ M epinephrine / 3 mM  $\text{CaCl}_2$  resulted in an increase in PAC1 binding on only the GPIb-normal (upper) subpopulation, whereas the GPIb-reduced (lower) subpopulation was unresponsive to agonist.

**FIGURE 12**

**FIGURE 13** Percent positive analysis for platelet surface P-selectin

Percent positive flow cytometric analysis for platelet surface P-selectin of fresh (n=8), Day 3/4 22°C liquid-preserved (n=5) and cryopreserved (n=8) platelet products. Platelets were identified with anti-CD41 antibody. Day 3/4 liquid-preserved platelets had higher background P-selectin (open bars) compared to fresh and were slightly less responsive to both 2 units/mL thrombin and a combination of 20  $\mu$ M ADP / 20  $\mu$ M epinephrine. The GPIb-normal subpopulation of the cryopreserved product had the highest background P-selectin and was responsive to both agonist challenges. The GPIb-reduced subpopulation of the cryopreserved product had lower background P-selectin than the GPIb-normal component and was completely unresponsive to agonist. Data are mean  $\pm$  SEM, \* indicates p-value < 0.05 compared to fresh.

FIGURE 13

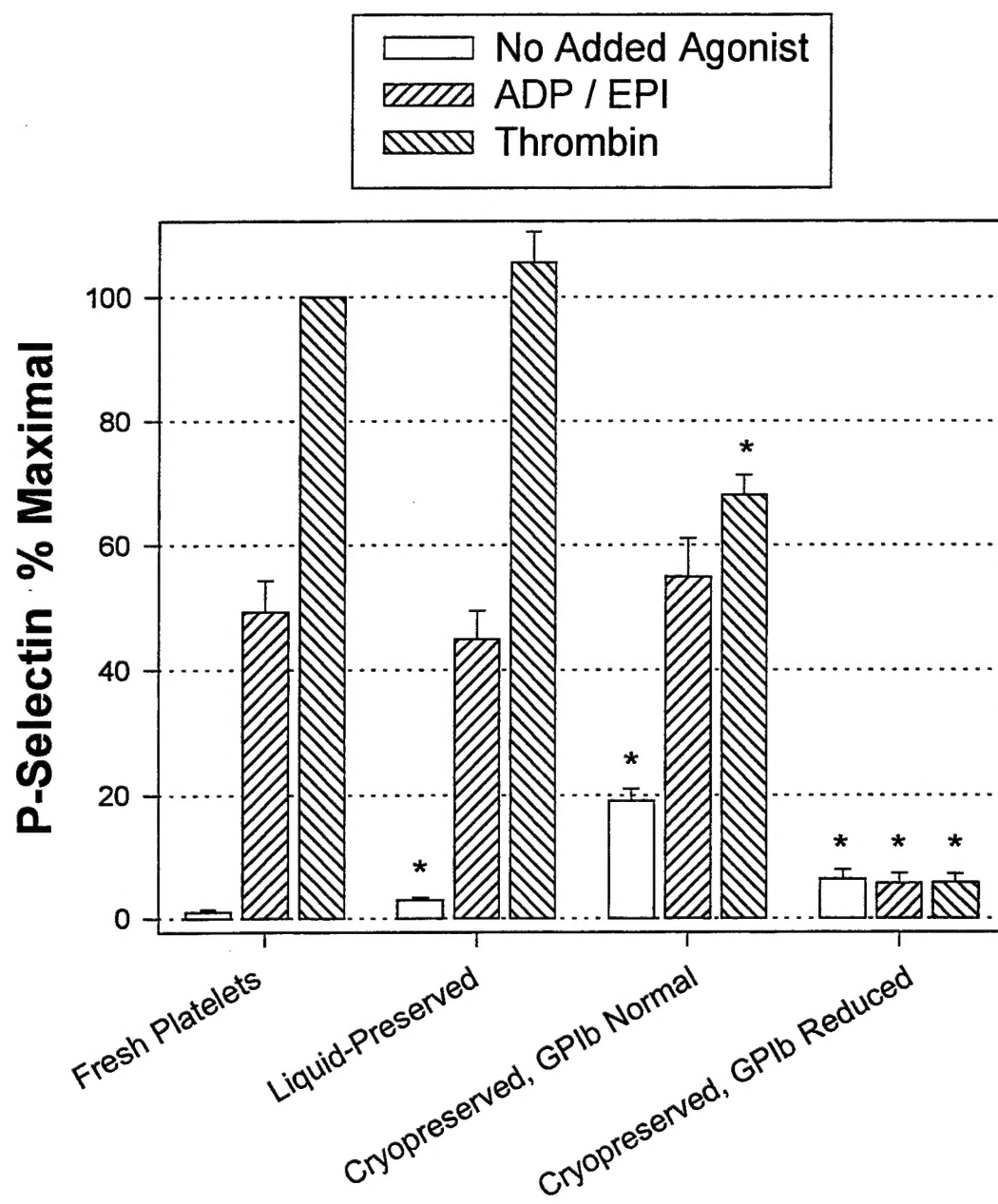


**FIGURE 14** Percent maximal fluorescence analysis for platelet surface P-selectin

Percent maximal fluorescence analysis for platelet surface P-selectin measured by flow cytometry. Platelets were identified with anti-CD41 antibody. Thrombin-activated fresh platelet (n=8) anti P-selectin antibody S12 fluorescence was assigned 100 units. The Day 3/4 22°C liquid-preserved platelets (n=5) had higher background levels of P-selectin (open bars) compared to fresh and had a similar responsiveness to both 2 units/mL thrombin and a combination of 20  $\mu$ M ADP / 20  $\mu$ M epinephrine. The GPIb-normal subpopulation of the cryopreserved product had the highest background P-selectin and was responsive to both agonist challenges, but did not express as much P-selectin with thrombin activation as fresh platelets. The GPIb-reduced subpopulation of the cryopreserved product had lower background binding of anti-P-selectin than the GPIb-normal component and was completely unresponsive to agonist. Data are mean  $\pm$  SEM, \* indicates p-value < 0.05 compared to fresh platelets.



FIGURE 14



**FIGURE 15** Representative contour plot histograms comparing liquid-preserved and cryopreserved platelets

Representative contour plot histograms comparing 3 day 22°C liquid-preserved (top row) and cryopreserved (bottom row) platelet products using a panel of antibodies and flow cytometry. In all cases platelets were identified with anti-CD41 antibody. Log-amplified forward light scatter is on the Y axis of all 8 histograms. The X axis of the 4 columns represents from left to right: anti factor V antibody binding, anti activated (FNG receptor) GPIIb-IIIa complex antibody binding, anti P-selectin antibody binding and anti GPIb antibody binding to the platelet surface. Cryopreserved platelets have increased levels of surface factor V, activated GPIIb-IIIa and P-selectin compared to the day 3/4 liquid preserved. Cryopreserved platelets have decreased levels of surface GPIb. For both GPIb and factor V, the cryopreserved platelets contain distinctly separable subpopulations binding variable levels of antibody.

FIGURE 15

